## Remarks

Applicants thank the Examiner for the helpful comments and the opportunity to interview the pending application on July 31, 2003, and the subsequent phone interviews. In view of these conversations, Applicants provide the following remarks.

Claims 33-132, 145-304, and 367-446 are pending. Claims 45-52, 65-72, 85-92, 105-112, 125-132, 157-164, 177-184, 197-204, 217-224, 237-244, 257-264, 277-284, 297-304, 379-386, 399-406, and 419-426 have been withdrawn. Claims 225, 245, 265, 285, 407, and 427 have been amended. Support in the specification for these amendments are described below. Applicants particularly note that these amendments are not intended to narrow, nor do they narrow, the scope of the claimed subject matter. These amendments have been made merely to accommodate the Examiner's objections based on minor formalities. Thus, no new matter has been added.

### *I. Formal Matters - Information Disclosure Statements*

Applicants thank the Examiner for acknowledging the information disclosure statements filed on August 16, 2002 and February 3, 2003. Paper No. 37, paragraphs 1A-B. Applicants bring to the attention of the Examiner that additional, supplemental Information Disclosure Statements were filed on February 8, 2000 and March 16, 2001, which appear to not have been considered by the Examiner. For the Examiner's convenience, Applicants provide copies of these Form PTO-1449 and Form PTO/SB/08A (hereinafter referred to as "Forms") for the Examiner's consideration. Applicants respectfully request that the Examiner review the references listed on the enclosed Forms and indicate consideration of these references by initialing and returning the Forms to Applicants.

### *II. Claim Objections*

The Examiner objected to claims 245, 285, 407, and 427 because the claim language could allegedly be improved. Paper No. 37, paragraph 4A. Applicants have amended claims 245, 285, 407, and 427 to recite "expressing a protein fragment of the protein encoded by the cDNA . . .". Applicants respectfully request that the objection to claims 245, 285, 407, and 427 be withdrawn.

*III. Double Patenting*

The Examiner maintained the *provisional* rejection of pending claims 33-44, 53-64, 73-84, 93-104, 113-124, 145-156, 165-176, 185-196, 205-216, 225-236, 245-256, 265-276, 285-296, 367-378, 387-398, 407-418, and 427-438 under the judicially created doctrine of obviousness-type double patenting over the claimed invention in the following copending U.S. Applications: 09/257,272, 09/935,726, and 09/107,997. Paper No. 37, paragraph 5A.

The Examiner also *provisionally* rejected claims 33-132, 145-304, and 367-446 under the judicially created doctrine of obviousness-type double patenting as being obvious over claim 18 of U.S. Application 10/060,523. Paper No. 37, paragraph 5B.

Applicants acknowledge these provisional rejections. Upon receipt of a notice of allowance in this or in one of the above-referenced applications, Applicants will file an appropriate disclaimer in the remaining applications, to the extent that such a disclaimer is necessary, or will cancel any conflicting claims that remain pending.

As for the claims pending in Serial No. 09/107,997 and 09/257,272, Applicants acknowledge that a clear line of demarcation will be maintained as set forth in M.P.E.P. § 822.

The Examiner also *provisionally* rejected claims 33-132, 145-304, and 367-446 under the judicially created doctrine of obviousness-type double patenting as being obvious over claims 38 and 42-71 of U.S. Application 09/499,468. Paper No. 37, paragraph 5C. Applicants note that claim 38 has been canceled and claim 42 has been amended in U.S. Application 09/499,468 to recite "a method of treating a patient having an injury to or degeneration of a photoreceptor cell" and to recite "a therapeutically effective amount" of the VEGF-2 protein. Neither of these claim amendments are disclosed or suggested by the claims recited in the above captioned application. Moreover, the process steps are not inherently taught by the above captioned application, since the treated patient must have "an injury to or degeneration of a photoreceptor cell." Therefore, the amendments to claim 42 in U.S. Application 09/499,468 should obviate the provisional double patenting rejection.

The Examiner also *provisionally* rejected claims 62-89 and 111-150 under the judicially created doctrine of obviousness-type double patenting as being obvious over claim 86 of U.S. Application 10/127,551. Paper No. 37, paragraph 5D.

Applicants acknowledge this provisional rejection. Upon receipt of a notice of allowance in this or in one of the above-referenced applications, Applicants will file an

appropriate disclaimer in the remaining application, to the extent that such a disclaimer is necessary, or will cancel any conflicting claims that remain pending.

***IV. Claim Rejections - Enablement***

The Examiner rejected claims 33-44, 53-64, 73-84, 93-104, 225-236, 245-256, 265-276, 285-296, 367-378, 407-418, and 427-438 under 35 U.S.C. § 112, first paragraph, as lacking enablement for two reasons.

First, the Examiner rejected the fragment claims (claims 225, 245, 265, 285, 407, and 427) because the specification allegedly does not enable fragments comprising SEQ ID NO:8 which have angiogenic activity. Paper No. 37, paragraph 6C. As discussed during the interviews, it was agreed that if the claims were amended to recite (1) a functional limitation, such as "endothelial cell proliferation" rather than "endothelial cell proliferative activity" and (2) that the fragment includes a region comprising the 8 cysteines, the rejection would be withdrawn. Papers 38-39. Consistent with the Examiner's request, Applicants have amended claims 265, 285 and 427 to recite "proliferates endothelial cells" and claims 225, 245, and 407 to recite "promotes angiogenesis." Additionally claims 225, 245, 265, 285, 407, and 427 have been amended to replace the reference to "SEQ ID NO:8" with "protein fragment comprises amino acids 108-188 of SEQ ID NO:2." Support for these amendments can be found in the specification, for example, at page 9, lines 21-25. Applicants believe that these amendments overcome the Examiner's rejection, and therefore, respectfully request that the rejection of the fragment claims (claims 225, 245, 265, 285, 407, and 427) under 35 U.S.C. § 112, first paragraph, be withdrawn.

Second, the Examiner rejected claims 33, 53, 73, 93, and 367 as lacking enablement because the specification allegedly does not enable "mature" or "proprotein" forms of SEQ ID NO:2 or 4. Paper No. 37, paragraph 6C. As indicated on the Interview Summary, Applicants' arguments presented previously and during the interviews have overcome this rejection. Papers 38-39. Therefore, Applicants respectfully request that the rejection of claims 33, 53, 73, 93, and 367 under 35 U.S.C. § 112, first paragraph, be withdrawn.

*V. Claim Rejections – Written Description*

The Examiner maintained the rejection of claims 33-44, 53-64, 73-84, 93-104, and 367-378 under 35 U.S.C. § 112, first paragraph, as lacking written description of the recitation of "mature" and "proprotein" because even though the mature and the proprotein forms "may be inherently formed in a given expression system, for example, the exact sequence is still not known." Paper No. 37, paragraph 7B. Thus, it appears that the PTO is requiring the amino acid sequence of the inherently produced mature and proprotein forms to be explictly disclosed in the specification in order for the claims to satisfy written description. Applicants continue to disagree with the Examiner's reasoning and traverse the rejection.

*A. Claim Terms Need to be Only as Definite as Art Requires*

Claim terms are to be given their broadest reasonable interpretation, consistent with the specification and consistent with the interpretation that one skilled in the art would reach. *In re Cortright,* 165 F.3d 1353, 1358 (Fed. Cir. 1999). There is a "heavy presumption" that terms used in claims have the ordinary meaning that would be attributed to those words by persons skilled in the relevant art. *See CCS Fitness, Inc. v. Brunswick Corp.*, 288 F.3d 1359, 1366, 62 USPQ2d 1658, 1662 (Fed. Cir. 2002); *K-2 Corp. v. Salomon S.A.*, 191 F.3d 1356, 1362-63, 52 USPQ2d 1001, 1004 (Fed. Cir. 1999); *Johnson Worldwide Assocs., Inc. v. Zebco Corp.*, 175 F.3d 985, 989, 50 USPQ2d 1607, 1610 (Fed. Cir. 1999); *Specialty Composites v. Cabot Corp.*, 845 F.2d 981, 986, 6 USPQ2d 1601, 1604 (Fed. Cir. 1988). Thus, a crucial step in determining the meaning of a technical claim term is to determine the ordinary meaning that would be ascribed by a person skilled in the relevant art.

Additionally, technical publications can be used to confirm the skilled artisan's definition of a technical claim term, as well as to show that the patentee intended to apply that definition. *See,* for example, *Arthur A. Collins, Inc. v. Northern Telecom Ltd.*, 216 F.3d 1042, 1045 (Fed. Cir. 2000) ("[w]hen prior art that sheds light on the meaning of a term is cited by the patentee, it can have particular value as a guide to the proper construction of the term, because it may indicate not only the meaning of the term to persons skilled in the art, but also that *the patentee intended to adopt that meaning.*") (emphasis added); *see also, In re Cortright; Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1584 (Fed. Cir. 1996). Although a general dictionary is sometimes consulted, the definition it provides "is secondary to the specific

meaning of a technical term as it is used and understood in a particular technical field." *Hoechst Celanese Corp. v. BP Chemicals Ltd.*, 78 F.3d 1575, 1580 (Fed. Cir. 1996).

Finally, when technical claim terms are used consistently with the prior art and as understood by the skilled artisan, there is no requirement that the patentee provide a definition of the term in either the specification or the claims. *Johnson Worldwide Associates, Inc. v. Zebco Corp.*, 175 F.3d 985, 990 (Fed. Cir. 1999); *In re Paulsen,* 30 F.3d 1475, 1480 (Fed. Cir. 1994); *Intellicall, Inc. v. Phonometrics, Inc.*, 952 F.2d 1384, 1387-8 (Fed. Cir. 1992) ("Where an inventor chooses to be his own lexicographer and to give terms uncommon meanings, he must set out his uncommon definition in some manner within the patent disclosure").

Thus, determining the meaning of a technical claim term must not be done in a vacuum, but always in light of the teachings of the prior art, the disclosure as it would be interpreted by one having of skill in the art, and the statements made during prosecution. *In re Moore,* 439 F.2d 1232, 1235 and n.2 (CCPA 1971).

### *B. Mature and Proprotein Forms are Defined in the Specification Consistently with the Art's Usage of these Terms.*

In the specification, "proprotein" and "mature" portions of VEGF-2 are specifically described as follows:

> The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Specification, page 11, lines 11-21. Additional description of the "mature" and "proprotein" forms can be found at page 9, lines 5-8 and page 10, lines 4-17. The lack of specified amino acid sequences in the specification of the "mature" and "proprotein" forms of VEGF-2 is not a

flaw of the specification. Rather, the technical terms "mature" and "proprotein" are being used consistently in the specification with how the terms are routinely used in the art.

The skilled artisan frequently refers to either the "mature" or "proprotein" forms of a protein without explicitly defining these forms by amino acid sequence. Evidence of this usage is illustrated in Exhibit A, where the authors refer to the "mature" and "proprotein" forms of VEGF-2 without defining, or even knowing, the complete amino acid sequence of these forms. See, for example, page 291, second column, second paragraph of Joukov et al., "A Novel Vascular Endothelial Growth Factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) Receptor Tyrosine Kinases" the Embo Journal, Vol. 15, No. 2, (1996)(herein included as Exhibit A.) It was not until a subsequent publication, attached as Exhibit B, did these same authors define the complete amino acid sequence of the "mature" and "proprotein" forms of VEGF-2. See, for example, the Abstract, and page 3898, column 2, last paragraph that extends to page 3899, and Figure 10 of Joukov et al., "Proteolytic Processing Regulates Receptor Specificity and Activity of VEGF-C," the Embo Journal, Vol. 16, No. 13, (1997)(herein included as Exhibit B.) Thus, when referring to the "mature" or "proprotein" forms of a protein, the skilled artisan does not require that the specific amino acid sequence be defined. The PTO is therefore improperly requiring a level of precision in technical claim terms that is inconsistent with how those terms are used in the art.

### *C. Amino Acid Sequence of "Mature" and "Proprotein" Forms are Routine to Identify*

The Examiner also appears to argue that without describing the amino acid sequence of the "mature" or "proprotein" forms, one would not "be able to identify these forms in order to isolate them." Paper No. 37, paragraph 7B, page 9, lines 1-3. However, contrary to the Examiner's position, it is routine to determine the amino acid sequence of the "mature" and/or the "proprotein" forms of a protein.

At the time of filing, it was well known that the full-length proteins of the PDGF/VEGF family undergo proteolytic processing to generate the mature form of the protein. See, for example, Exhibit B, page 3906, first column, last sentence. Moreover, at the time of filing it was well known how to N-terminally sequence isolated proteins from cells transfected with a particular gene sequence. Therefore, if necessary, the skilled artisan could have routinely

expressed the VEGF-2 sequence disclosed in the specification and N-terminally sequence the isolated protein, thereby identifying the amino acid sequence of the mature form.

### *D. No Evidence that "Mature" and "Proprotein" Forms will be Differentially Produced*

The Examiner also argued that "the structure of a 'mature form of a polypeptide' cannot be predicted on the basis of the amino acid sequence of the entire protein since the protein may be proteolytically cleaved in vivo, as well as being differentially processed based on which in [sic] tissue the protein is expressed." Paper No. 37, page 8, lines 24-27. However, the Examiner has not provided any evidence to support this position.

Applicants have herein provided a third party publication that demonstrates VEGF-2 is in fact processed similarly in different cell types. When the authors of Exhibit B expressed VEGF-2 in a variety of host cells, they found that VEGF-2 proteolytically processed "similarly in different cell types." See, Exhibit B, page 3901, second column, last sentence of first complete paragraph. Thus, unless the Examiner provides contradictory evidence, there is no basis on which to argue that VEGF-2 processes differently depending on the cell type.

However, even if the "mature" form of VEGF-2 did process differently depending on the cell type, the claims still would not lack written description. In a case with facts analogous to the present situation, the Federal Circuit recently held that claims directed to proteins produced from a "vertebrate" or "mammalian" host cell satisfied the written description requirement, even though there might be "minor differences" in applying the disclosed methods to any other type of host cell and that those of ordinary skill in the art in 1984 could have "easily" figured out the differences in methodology. Amgen, Inc. v. Hoechst Marion Roussel, Inc., 315 F.3d 1313, 1331, 65 U.S.P.Q.2d 1385 (Fed. Cir. 2003). Thus, even if processing of VEGF-2 is host cell specific, the claims directed to the "mature" and "proprotein" forms of VEGF-2 are fully supported by the specification.

### *E. Processed Forms are Inherently Produced by Cells*

As acknowledged by the Examiner, the "mature" and "proprotein" forms of VEGF-2 are inherently formed in a given expression system and therefore, are inherent in structure. Paper No. 37, paragraph 7B, page 8, lines 18-19 and 29. It is well accepted that by "disclosing in a patent application a device that *inherently* performs a function or has a property, operates

according to a theory or has an advantage, a *patent application necessarily discloses* that function, theory or advantage, even though it says nothing explicit concerning it." M.P.E.P. § 2163.07(a). Therefore, as permitted by M.P.E.P. § 2163.07(a), Applicants are prepared to amend the specification to explicitly refer, by amino acid sequence, to the inherently produced "mature" and "proprotein" forms of VEGF-2. Applicants specifically request clarification as to whether amending the specification to include these inherently produced sequences will overcome the outstanding rejection.

***F. Standard for Written Description***

The function of the "written description" requirement of 35 U.S.C. 112, first paragraph, is to ensure that applicants had possession of the claimed subject matter, as of the filing date of application relied on. *In re Blaser*, 556 F.2d 534, 194 USPQ 122 (CCPA 1977). The inquiry into satisfaction of the written description requirement is factual and depends on the nature of the invention and the amount of knowledge imparted to those of skill in the art by the disclosure. *In re Wertheim*, 646 F.2d 527, 191 USPQ 90 (CCPA 1976). Satisfaction of the "written description" requirement does not require *in haec verba* antecedence in the originally filed application. *In re Lukach*, 440 F.2d 1263, 169 USPQ 795 (CCPA 1971). The written description requirement can be satisfied by showing that the disclosed subject matter, when given its 'necessary and only reasonable construction,' inherently (*i.e.*, necessarily) satisfies the limitation in question. *Staehelin v. Secher,* 24 USPQ2d, 1513, 1520 (Bd. Pat. Int'f. 1992) ("a specification need not describe the exact details for preparing every species within the genus described"). In general, precedent establishes that although the applicant 'does not have to describe exactly the subject matter claimed, the description must clearly allow persons of skill in the art to recognize that [the applicant] invented what is claimed.' *In re Gosteli,* 872 F.2d 1008,1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

To overcome a prima facie case of unpatentability under 35 U.S.C. §112, first paragraph, the applicants must show by evidence or argument that the invention as claimed is adequately described to one of ordinary skill in the art. *In re Alton* 76 F.3d 1168, 1175 (Fed. Cir. 1996). The arguments and exhibits provided in this response, as well as those that have been made previously in the Response dated February 3, 2003 (herein incorporated by reference in its entirety) provides evidence that one of skill in the art would recognize that the mature form of VEGF-2 is adequately described by the instant application. If a person of ordinary skill in the

art would have understood the inventor to have been in possession of the claimed invention at the time of filing, even if every nuance of the claim is not explicitly described in the specification, then the adequate written description requirement is met. *In re Alton* 76 F.3d 1168, 1175 (Fed. Cir. 1996). The Federal Circuit has noted that the priority application need not use the identical words to describe the claimed invention, if it shows the subject matter claimed with an adequate direction as to how to obtain it. *Kennecott v. Kyoura International, Inc.* 835 F.2d 1419. 1422, 5 USPQ 2d 1194, 1197 (Fed. Cir. 1987), *cert denied*, 486 U.S. 1008 (1988).

In this instance, the priority application and the instant application clearly describe the subject matter of the invention and also provide adequate direction as to how to obtain the mature form of VEGF-2. Furthermore, one of ordinary skill in the art would recognize that the applicants were in possession of the claimed invention. Indeed, it is unnecessary for the specification to explicitly define by amino acid sequence the beginning and end of the processed, mature form of VEGF-2 in order for one skilled in the art to recognize a "mature portion of a protein."[1] The specification teaches the "mature portion" of VEGF-2 because the "mature portion" of VEGF-2 is naturally and inherently produced when expressed by a host cell.

Thus, the instant specification, and the specification of the priority application, contains sufficient information required of one of ordinary skill in the art to recognize that the applicants were in possession of the invention as claimed. Hence, the rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

**Conclusion**

In view of the foregoing remarks Applicants believe they have fully addressed the Examiner's concerns and that this application is now in condition for allowance. An early notice to that effect is urged. A request is made to the Examiner to call the undersigned at the phone number provided below if any further action by Applicants would expedite allowance of this application.

[1] However, if this is required, Applicants are prepared to amend the specification to include the amino acid sequence of the "mature" and "proprotein" forms of VEGF-2 as provided by M.P.E.P § 2163.07(a).

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

Dated: October 15, 2003

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Enclosures
MMW/MJP/lc

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# A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases

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**Angiogenesis, the sprouting of new blood vessels from pre-existing ones, and the permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two known receptors Flt1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2). The Flt4 receptor tyrosine kinase is related to the VEGF receptors, but does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. In this study, we have purified the Flt4 ligand, VEGF-C, and cloned its cDNA from human prostatic carcinoma cells. While VEGF-C is homologous to other members of the VEGF/platelet derived growth factor (PDGF) family, its C-terminal half contains extra cysteine-rich motifs characteristic of a protein component of silk produced by the larval salivary glands of the midge, *Chironomus tentans*. VEGF-C is proteolytically processed, binds Flt4, which we rename as VEGFR-3 and induces tyrosine autophosphorylation of VEGFR-3 and VEGFR-2. In addition, VEGF-C stimulated the migration of bovine capillary endothelial cells in collagen gel. VEGF-C is thus a novel regulator of endothelia, and its effects may extend beyond the lymphatic system, where Flt4 is expressed.**

*Keywords*: angiogenesis/endothelium/growth factor/lymphatic system/VEGF

## Introduction

The development of blood vessels from early (*in situ*) differentiating endothelial cells is termed vasculogenesis (Risau and Lemmon, 1988). The formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis (Folkman, 1995). Vascular endothelial cells can give rise to several types of functionally and morphologically distinct vessels and when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases (Risau, 1995). Angiogenesis also plays a major role in pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases and tumour growth and metastasis (Folkman, 1995).

Angiogenesis is regulated by a balance between angiogenic factors and inhibitors which bind to specific receptors on target cells. Five endothelial cell-specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4, Tie and Tek/Tie-2, have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction (for reviews, see Mustonen and Alitalo, 1995; Shibuya, 1995). Targeted mutations inactivating Flt-1, Flk-1, Tie and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level (Dumont *et al.*, 1994; Millauer *et al.*, 1994; Fong *et al.*, 1995; Puri *et al.*, 1995; Sato *et al.*, 1995; Shalaby *et al.*, 1995). VEGFR-1 and VEGFR-2 bind VEGF with high affinity ($K_d$ 16 pM and 760 pM, respectively) (de Vries *et al.*, 1992; Terman *et al.*, 1992; Millauer *et al.*, 1993; Waltenberger *et al.*, 1994) and VEGFR-1 also binds the related placenta growth factor (PlGF; $K_d$ ~200 pM) (Maglione *et al.*, 1993; Park *et al.*, 1994), while the ligands for Tie, Tek and Flt4 have not yet been reported.

We report isolation of a novel vascular endothelial growth factor and its cloning from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. We show that the isolated cDNA encodes a protein which is proteolytically processed, secreted to cell culture medium, binds to the extracellular domain of Flt4 and induces tyrosine autophosphorylation of Flt4 and VEGFR-2. VEGF-C also stimulates the migration of endothelial cells in collagen gels.

## Results

### *Identification, purification and N-terminal sequencing of the Flt4 ligand*

In order to find a source for the Flt4 ligand, we screened conditioned media (CM) from human tumour cell cultures for their ability to stimulate the Flt4 receptor. Serum-free medium conditioned for 5 days with PC-3 prostatic adenocarcinoma cells was found to stimulate tyrosine phosphorylation of Flt4 expressed in transfected NIH 3T3 cells (Figure 1, lanes 1–3). The stimulating activity was increased upon concentration of CM by ultrafiltration through a 10 kDa cut-off membrane (lanes 2, 3 and 6). Pretreatment with the extracellular domain of Flt4 (Flt4EC) covalently bound to Sepharose completely abolished the ability of CM to stimulate tyrosine phosphorylation of Flt4 (lanes 3–5). No autophosphorylation of Flt4 was detected when transfected cells were treated with purified VEGF or PlGF (Pajusola *et al.*, 1994 and data not shown). These data indicated that the PC-3 cells produce a soluble ligand which binds to the extracellular domain of Flt4 and activates this receptor.

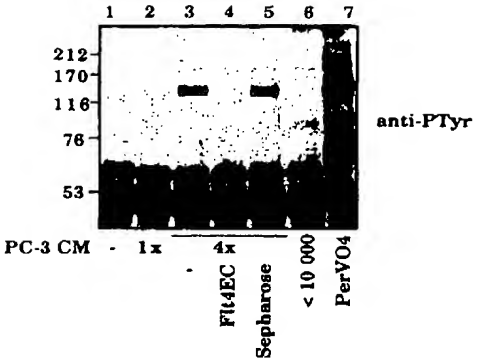



Fig. 1. Identification of the Flt4 ligand from PC-3 cell CM. Flt4-expressing NIH 3T3 cells were incubated with PC-3 cell CM, lysed and the lysates were immunoprecipitated with Flt4-specific antiserum followed by SDS–PAGE, Western blotting and detection using anti-phosphotyrosine (anti-PTyr) antibodies. Lane 1, unconditioned medium. Lane 2 shows weak phosphorylation of a band of 125 kDa upon stimulation with unconcentrated PC-3 CM. The 125 kDa band comigrated with the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7). Lane 3, stimulation with PC-3 CM concentrated 4-fold using Centricon-10 device (Amicon). Lanes 4 and 5, stimulation after treatment of the concentrated PC-3 CM with 30 μl of the recombinant Flt4EC coupled to Sepharose or with unsubstituted Sepharose respectively. Lane 6, Centricon 10 flow-through containing proteins of <10 kDa molecular mass.

The Flt4-stimulating activity was concentrated from PC-3 CM (Figure 2A, lanes 1–3) and used to purify the ligand by affinity chromatography on Flt4EC (lanes 4–11). The Flt4-stimulating material was eluted at pH 2.4 (lanes 8 and 9). Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. As shown in Figure 2B, a major polypeptide having a molecular mass of ~23 kDa (asterisk, lane 6) and a minor one of 32 kDa were detected only in the fractions containing Flt4-stimulating activity, whereas the other polypeptide bands were major components of the starting material. N-terminal amino acid sequence analysis of the 23 kDa band gave the sequence $NH_2$-XEETIKFAAAHYN-TEILK-COOH.

### *Cloning of the Flt4 ligand from a PC-3 cDNA library*

Degenerate oligonucleotides designed on the basis of the N-terminal sequence of the isolated Flt4 ligand were used as primers in PCR to amplify cDNA encoding the N-terminal peptide from a PC-3 cell cDNA library (see Figure 3A and Materials and methods for details). The product of the expected size was cloned and sequenced and new primers were designed for amplification of the entire 5′ cDNA. The resulting PCR fragment was used as a probe to screen the PC-3 cell cDNA library. The two longest clones of 2.0 and 1.8 kb contained an open reading frame (ORF) of 350 residues shown in Figure 3B, having two possible methionine codons (marked in bold) for translational initiation and a putative secretory signal peptide (underlined) followed by the N-terminal sequence of the purified Flt4 ligand (marked in bold).

### *Flt4 ligand is a novel member of the PDGF family, VEGF-C*

Comparison with the amino acid sequences of growth factors of the VEGF/PDGF family shows that all eight cysteine residues typical for members of this family (Heldin *et al.*, 1993), as well as several other residues are conserved in Flt4 ligand (Figure 3B). Thus, the Flt4 ligand is a novel member of the VEGF family of growth factors, which we have designated VEGF-C. Homologous portions of VEGF-C are ~30% identical to $VEGF_{165}$ (Leung *et al.*, 1989), ~27% to $VEGF\text{-}B_{167}$ (Olofsson *et al.*, 1996), ~25% to PlGF-1 (Maglione *et al.*, 1991) and ~22–24% to PDGF-A and PDGF-B (Betsholtz *et al.*, 1986). However, the VEGF-C polypeptide continues with sequences rich in cysteine residues, some of which can be aligned with the C-terminus of $VEGF_{165}$ as shown in Figure 3B. Interestingly, the C-terminal cysteine residues of VEGF-C occur in repeat units typical for the Balbiani ring 3 protein (BR3P), a major cysteine-rich protein of the larval saliva of the the midge, *Chironomus tentans* (Dignam and Case, 1990; Paulsson *et al.*, 1990). Three repeats, of 24 residues each, are followed by a shorter repeat of 19 residues (Figure 3C), all conforming to the most common type of repeat in BR3P (~40% identity with amino acid sequence 1244–1371) (Paulsson *et al.*, 1990).

### *Recombinant VEGF-C is proteolytically processed and activates the Flt4 receptor tyrosine kinase*

The predicted molecular mass of the secreted polypeptide deduced from the VEGF-C ORF, 35.881 kDa suggests that VEGF-C mRNA may be first translated into a precursor, from which the mature ligand of 23 kDa is derived by proteolytic cleavage. Indeed, a putative precursor polypeptide with an apparent molecular mass of 32 kDa was bound to the Flt4EC affinity matrix from the CM of metabolically labelled cells transfected with a VEGF-C expression vector (Figure 4A). Increased amounts of a 23 kDa receptor binding polypeptide accumulated in the culture medium during a subsequent chase period of 3 h, but not thereafter (lanes 2–4 and data not shown), suggesting that the 23 kDa form is produced by proteolytic processing, which is cell-associated and incomplete, at least in the transiently transfected cells. In non-reducing conditions, higher molecular mass forms were seen, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers (arrows in Figure 4B). The CM of the transfected cells also stimulated Flt4 autophosphorylation (Figure 4C, lanes 1 and 2), but when the CM was pre-absorbed with the Flt4EC, no phosphorylation was obtained (lane 3). On the basis of these results and the above nomenclature, we have renamed Flt4 as VEGFR-3.

### *Stimulation of VEGFR-2 autophosphorylation by VEGF-C*

CM from 293 EBNA cells transfected with the VEGF-C vector was also used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2 (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). The cells were lysed and immunoprecipitated using VEGFR-2-specific antiserum (Waltenberger *et al.*, 1994).

The results of the experiment are presented in Figure 5A. A basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-

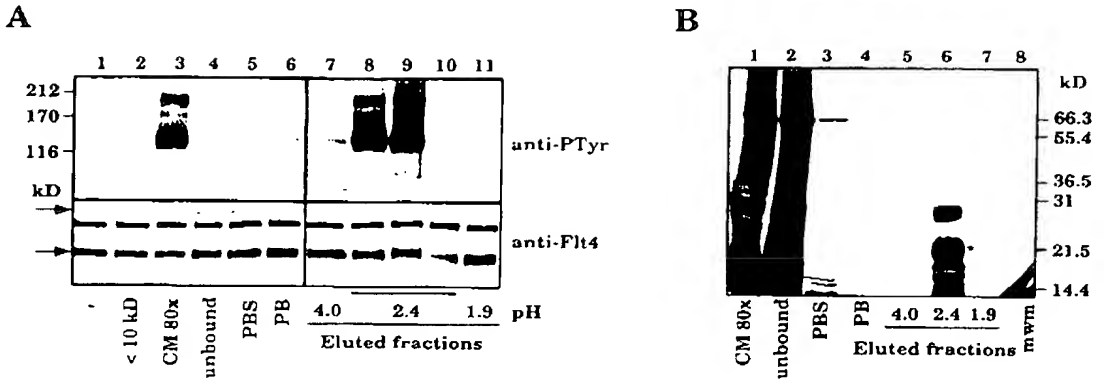



Fig. 2. Purification of the Flt4 ligand. (**A**) Flt4-expressing cells were treated with non-conditioned medium (lane 1), PC-3 cell CM or with different chromatographic fractions and Flt4 was immunoprecipitated and analysed in SDS–PAGE followed by Western blotting and detection with PTyr antibodies or Flt4-specific antiserum and the ECL method. The phosphorylated unprocessed 195 kDa and proteolytically processed 125 kDa forms of Flt4 (Pajusola *et al.*, 1994) are marked by arrows. Note that the (presumably intracellular) 175 kDa precursor of Flt4 is not phosphorylated upon stimulation. (**B**) Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. The designations of the lanes are as follows: <10 kDa and CM 80×, filtrate and retained fractions, respectively, obtained after concentration of CM by ultrafiltration through a 10 kDa cut-off membrane; unbound, CM after absorption with FLT4EC; PBS and PB, washes of the affinity matrix with phosphate buffered saline and phosphate buffer pH 6.8, respectively; 4.0, 2.4 and 1.9, fractions eluted from the affinity matrix at indicated pHs; mwm, molecular mass markers.

transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation (lanes 1 and 2). CM containing recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM (lanes 3–5). Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix (compare lanes 1, 5 and 6). The maximal effect of VEGF-C in this assay was comparable with the effect of recombinant VEGF added to the unconditioned medium at a concentration of 50 ng/ml (lane 8). Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2 (compare lanes 7 and 8). These results suggest that the VEGF-C expression vector encodes a ligand not only for VEGFR-3, but also for VEGFR-2.

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analysed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor β (PDGFR-β) which is abundantly expressed on fibroblastic cells. As can be seen from Figure 5B, a weak tyrosine phosphorylation of PDGFR-β was detected upon stimulation of Flt4-expressing NIH 3T3 cells with CM from the mock-transfected cells (compare lanes 1 and 2). A similar low level of PDGFR-β phosphorylation was observed when the cells were incubated with CM from the VEGF-C-transfected cells, with or without prior treatment with Flt4EC (lanes 3 and 4). In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR-β (lane 5).

### *VEGF-C stimulates endothelial cell migration in collagen gels*

CM from cell cultures transfected with the VEGF-C expression vector was placed in a well which was made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel assay described in Materials and methods. After 6 days of treatment, the cultures were stained and cells at different distances outside of the original ring of attachment were counted using fluorescent nuclear staining and detection with a fluorescence microscope equipped with a grid. A comparison of the numbers of migrating cells in successive 0.5×0.5 mm areas is shown in Figure 6A. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. An example of typical phase contrast and fluorescent microscopic fields of cultures stimulated with medium from mock-transfected or VEGF-C-transfected cells is shown in Figure 6B. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared with the stimulation by CM from VEGF-transfected cells (data not shown).

### *VEGF-C is expressed in multiple tissues*

Northern blotting and hybridization analysis showed that a 2.4 VEGF-C mRNA is present in the HT-1080 fibrosarcoma and PC-3 prostatic adenocarcinoma cell lines (Figure 7A). The 2.4 kb RNA and smaller amounts of a 2.0 kb mRNA were seen in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine (Figure 7B). Very little VEGF-C RNA was seen in the brain, liver or thymus and peripheral blood leukocytes (pbl) appeared negative.

## Discussion

Our results show that VEGFR-3 transmits signals for a novel growth factor. This conclusion is based on the specific binding of VEGF-C to recombinant Flt4EC protein and the induction of VEGFR-3 autophosphorylation by medium from VEGF-C-transfected cells. In contrast,

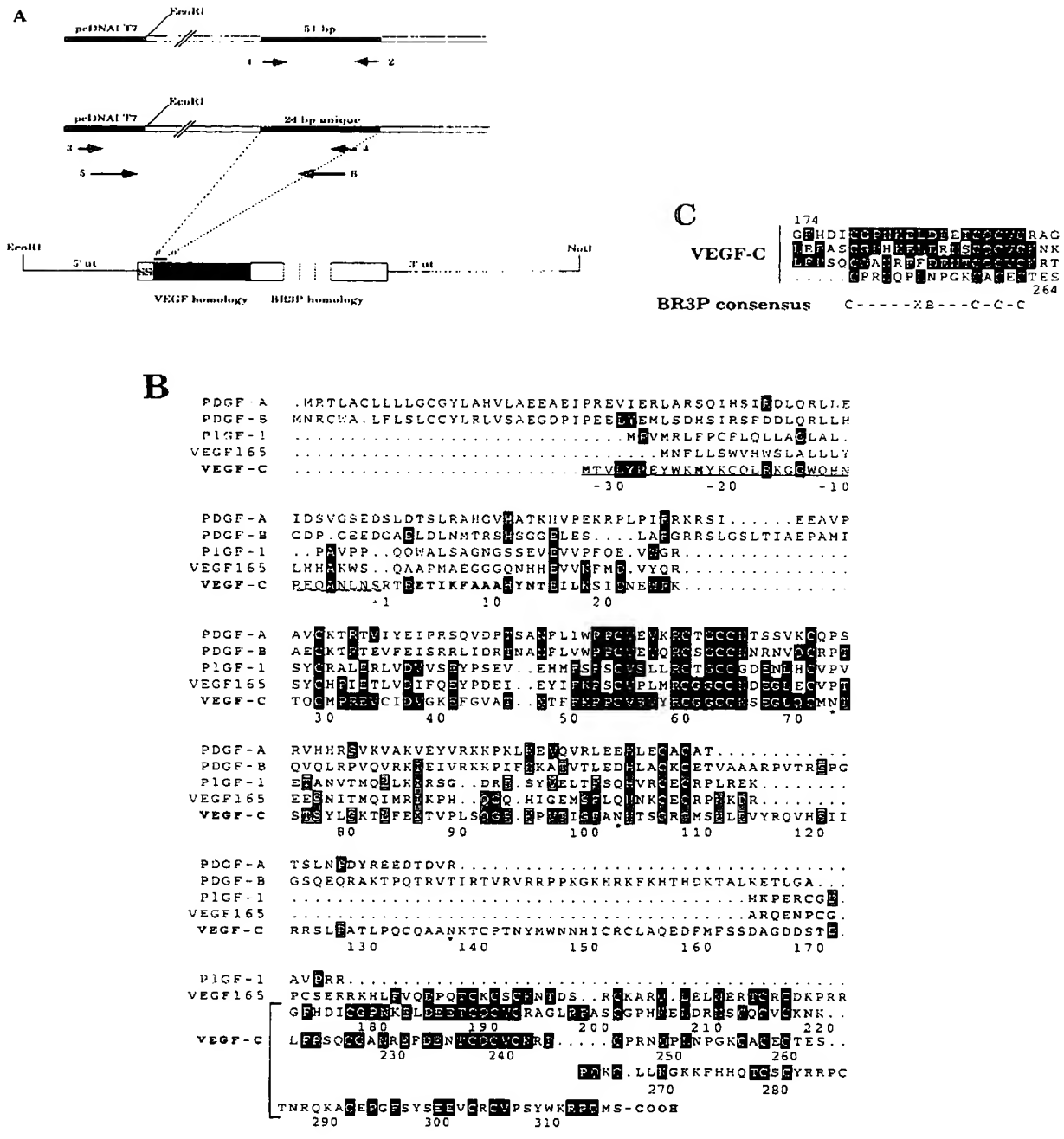



Fig. 3. Cloning and analysis of the Flt4 ligand (VEGF-C). (A) Schematic illustration of the PCR cloning of Flt4 ligand cDNA from PC-3 cDNA library. The primers and conditions used are detailed in Materials and methods. (B) Comparison of the amino acid sequence of VEGF-C with other growth factors of the VEGF/PDGF family (Betsholtz *et al.*, 1986; Leung *et al.*, 1989; Maglione *et al.*, 1991). VEGF-C amino acid residues are numbered beginning from the N-terminus after cleavage of the signal sequence. The PileUp program of Genetics Computer Group was used for alignment of the VEGF-homologous domains. The C-terminal motifs were aligned on basis of the pattern of cysteine residues. Three putative N-linked glycosylation sites (N-X-S/T) have been marked with asterisks. (C) Alignment of the repeated C-terminal motifs of VEGF-C with the consensus sequence of BR3P. B = D or N residue. X = non-polar or tyrosyl residue.

VEGF or PlGF did not show specific binding to VEGFR-3 or induce its autophosphorylation (Pajusola *et al.*, 1994).

Interestingly, the VEGF-C ORF is 350 amino acid residues long and our N-terminal sequence analysis confirmed that its putative signal sequence is removed before secretion. Glutamic acid was the second residue obtained in the N-terminal sequence analysis of the isolated protein, while the first residue could not be determined. According

to the deduced amino acid sequence of the VEGF-C cDNA this first residue is threonine. However, on the basis of the consensus residues surrounding signal sequence cleavage sites (von Heijne, 1986), the first residue following the signal sequence would be arginine, which may have been removed from the polypeptide after an additional proteolytic cleavage between arginine and threonine residues (see Figure 3B).

A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the VEGF-C encoded by the ORF may be due to proteolytic removal of sequences in the C-terminal region of the latter. Proteolytic processing of the VEGF-C precursor may occur at more than one cleavage site because the molecular mass of the recombinant secreted ligand, 32 kDa, was also less than the deduced molecular mass of the VEGF-C ORF without the signal peptide (see Figure 4A). By extrapolation from studies of the structure of PDGF (Heldin *et al.*, 1993), one can speculate that the region critical for receptor binding and activation by VEGF-C is contained within the first 180 or so amino acid residues of VEGF-C. Thus, the 23 kDa polypeptide binding VEGFR-3 is likely to represent the VEGF-homologous domain, which may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence.

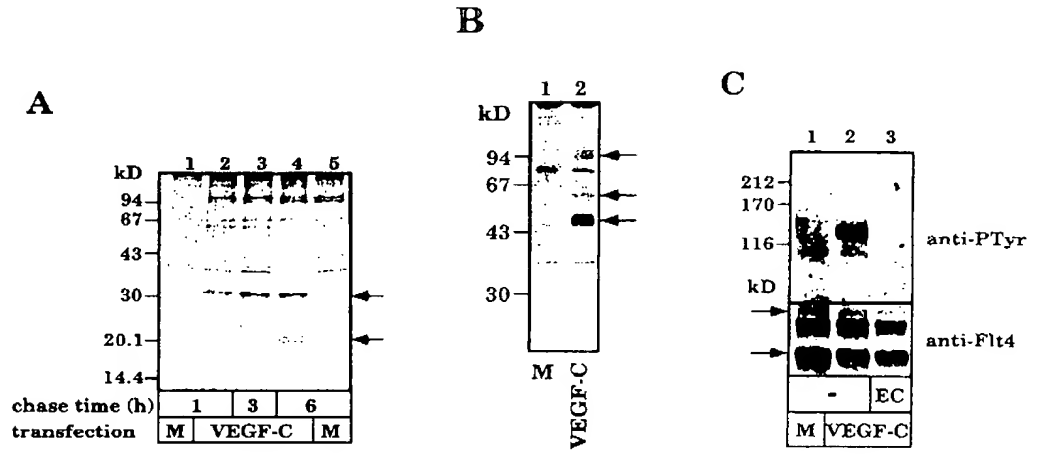



**Fig. 4.** Identification, dimerization and activity of recombinant VEGF-C. (**A**) Pulse–chase analysis of VEGF-C secreted by transfected cells. 293 EBNA cells were metabolically labelled using $^{35}$S-labelled methionine and cysteine mixture for 2 h and then chased in non-radioactive medium for the indicated periods of time. The medium was collected and VEGF-C was bound to Flt4EC–Sepharose followed by alkylation, SDS–PAGE and autoradiography. M = mock-transfected cells. Arrows indicate the 32 kDa and 23 kDa polypeptides of secreted VEGF-C. (**B**) VEGF-C isolated using Flt4EC–Sepharose after a 4 h continuous metabolic labelling was analysed under non-reducing conditions. The arrows indicate putative dimeric forms. (**C**) Stimulation of VEGFR-3 autophosphorylation by VEGF-C. NIH 3T3 cells expressing VEGFR-3 were stimulated with medium conditioned by cells transfected with VEGF-C cDNA. The medium was either untreated (lane 2) or treated with Flt4 EC (lane 3). Arrows indicate the phosphorylated forms of Flt4 (see the legend of Figure 2A).

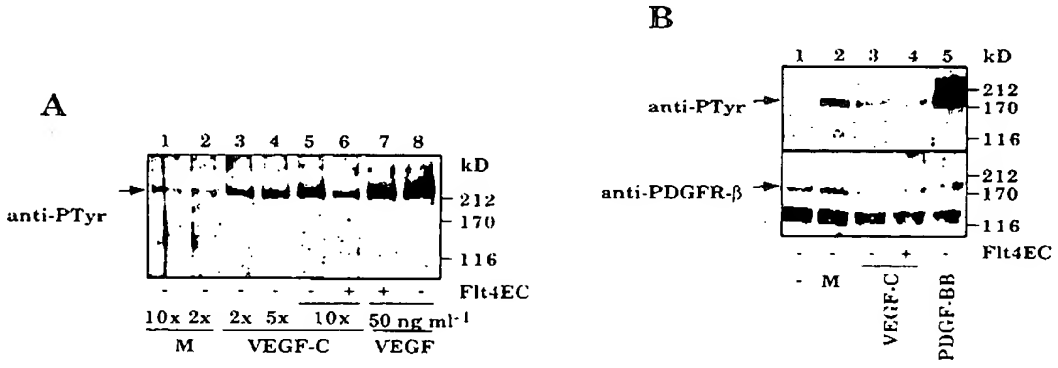
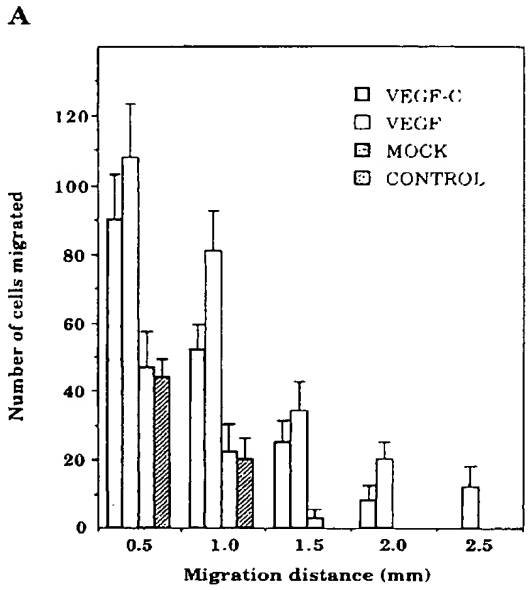



**Fig. 5.** VEGF-C stimulates autophosphorylation of VEGFR-2 (KDR) but has no effect on PDGFR-β phosphorylation. (**A**) PAE cells expressing VEGFR-2 were stimulated with 10- or 2-fold concentrated medium from mock-transfected 293 EBNA cells (lanes 1 and 2), or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C (lanes 3–6). VEGFR-2 was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the stimulations were carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF (lanes 7 and 8). Lanes 6 and 7 show stimulation with VEGF-C or VEGF containing media pretreated with Flt4EC. (**B**) Flt4-expressing NIH 3T3 cells were stimulated with non-conditioned medium (lane 1), 5-fold concentrated CM from mock-transfected (lane 2) or VEGF-C-transfected (lanes 3 and 4) cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB (lane 5). Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR-β was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR-β.



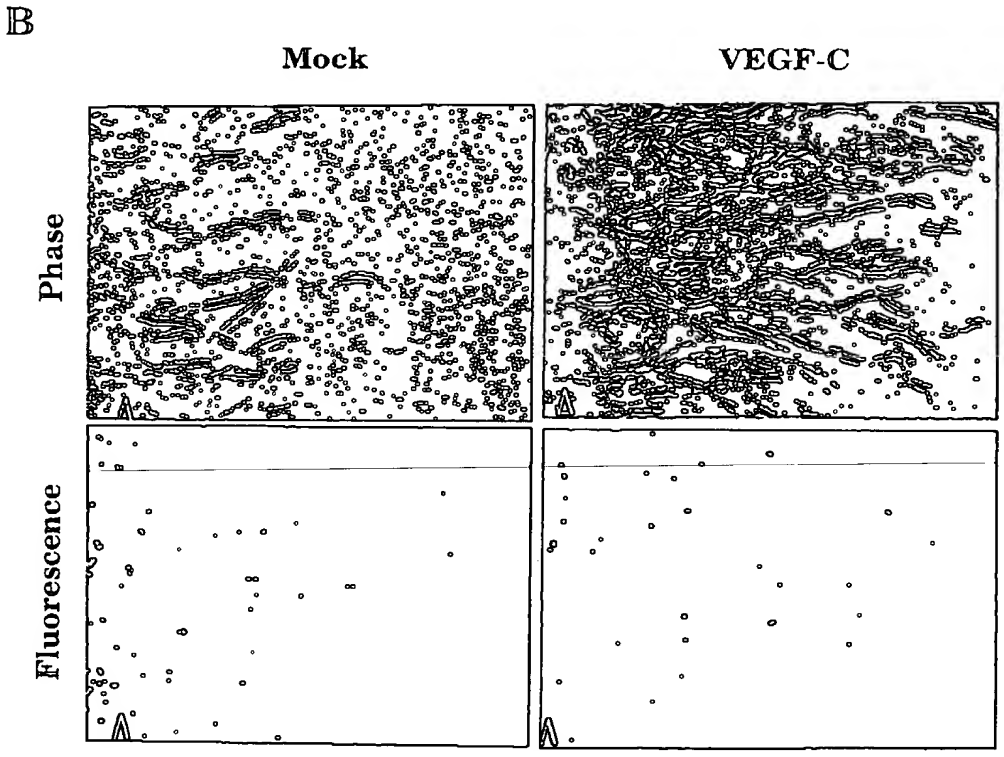




Fig. 6. VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay. (A) The diagram shows a comparison of the number of cells migrating at different distances from the original area of attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock: VEGF-C: VEGF) cells, 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5×0.5 mm squares using a microscope ocular lens grid and 10× magnification. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice in duplicate with similar results, and medium values from the one of the experiments are presented with the standard error bars. (B) Phase-contrast microscopy and fluorescent microscopy of the nuclear staining of BCE cells migrating towards the wells containing media conditioned by the mock-transfected cells or by VEGF-C-transfected cells. The areas shown are ~1×1.5 mm and arrows indicate the borders of the original ring of attachment.

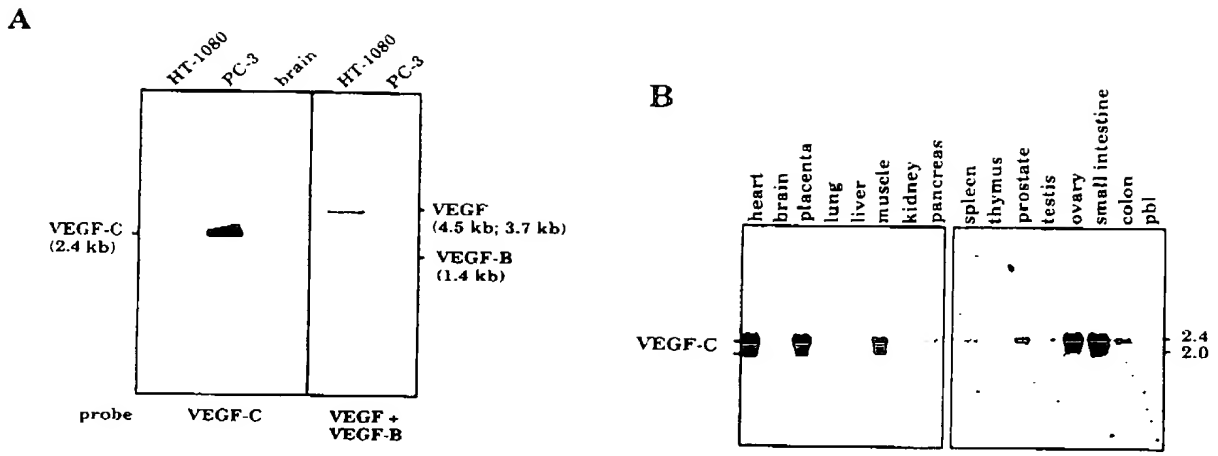



**Fig. 7.** Expression of VEGF-C mRNA in tumour cell lines and in human adult tissues. Northern blots containing 8 μg of isolated poly(A)$^+$ RNA from HT-1080 and PC-3 human tumour cells (A) and multiple human tissues (B, blot from Clontech) were probed with radioactively labelled insert of the 2.0 kb VEGF-C cDNA clone. Shown in (A) are also the 4.5 kb and 3.7 kb mRNA signals for VEGF and the 1.4 kb signal for VEGF-B (Olofsson *et al.*, 1996) in the same samples. Note that the tumour cell lines contain mainly mRNA of the 2.4 kb form.

The C-terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family show a pattern of spacing of cysteine residues reminiscent of the BR3P sequence (Dignam and Case, 1990; Paulsson *et al.*, 1990). This novel C-terminal silk-protein-like structural motif of VEGF-C may fold into an independent domain, which, on the basis of the considerations above, is at least partially cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the C-terminus of VEGF. In our experiments both the putative precursor and cleaved ligand were detected in the cell culture medium, although processing was apparently cell-associated on the basis of the pulse–chase experiments. The determination of the N-terminal sequence of the isolated C-terminal fragment will allow the identification of the proteolytic processing site. On the other hand, the generation of antibodies against different parts of the VEGF-C molecule will allow the exact determination of the precursor–product relationship and ratio, their cellular distribution and the kinetics of processing and secretion.

We have recently cloned another factor structurally homologous to VEGF, designated accordingly as VEGF-B (Olofsson *et al.*, 1996). Both of these factors share a conserved pattern of eight cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds creating an antiparallel dimeric biologically active molecule, similar to PDGF (Andersson *et al.*, 1992; Oefner *et al.*, 1992). Mutational analysis of the cysteine residues involved in the interchain disulfide bridges have shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity (Pötgens *et al.*, 1994). Putative dimers were evident in the analysis of VEGF-C under non-reducing conditions. It will be interesting to analyse these possible dimerization patterns of VEGF-C, but this is made technically difficult by the presence of precursor and processed forms and the high cysteine content of VEGF-C, which causes anomalous migration in gel electrophoresis under non-reducing conditions.

VEGFR-3, which thus distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2 (Pajusola *et al.*, 1992; Finnerty *et al.*, 1993; Galland *et al.*, 1993). However, the mature form of VEGFR-3 differs from the two other VEGFRs in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Pajusola *et al.*, 1994). Another difference is that the 4.5 and 5.8 kb VEGFR-3 mRNAs encode polypeptides differing in their C-termini and apparently in their signalling properties due to the use of alternative 3′ exons (Pajusola *et al.*, 1993; Borg *et al.*, 1995).

Besides VEGFR-3, VEGFR-2 tyrosine kinase also was shown to be activated in response to VEGF-C. VEGFR-2-mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of PAE cells overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity (Waltenberger *et al.*, 1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH 3T3 fibroblastic cells, but not in PAE cells (Pajusola *et al.*, 1994). Consistent with such results, the bovine capillary endothelial cells, which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs (our unpublished data), showed enhanced migration when stimulated with VEGF-C. Light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells, but such data must be confirmed by more detailed analyses of cell proliferation and survival in the presence and absence of specific factors. The existing data thus indicate that the VEGF ligands and receptors show a great specificity in their signalling, which may be cell type dependent.

The expression pattern of the VEGFR-3 (Kaipainen *et al.*, 1995) suggests that VEGF-C may function in the

formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues further suggests that this gene product is also involved in the maintenance of the differentiated functions of the lymphatic endothelium where VEGFR-3 is expressed (Kaipainen *et al.*, 1995). Lymphatic capillaries do not have well formed basal laminae and an interesting possibility remains that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but decreased in adult tissues (Millauer *et al.*, 1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect in lymphatic endothelium and a more redundant function shared with VEGF in angiogenesis and possibly permeability regulation of several types of endothelia.

Taken together these results show an increased complexity of signalling in the vascular endothelium. They reinforce the concept that when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases in several types of functionally and morphologically distinct vessels. However, upon suitable angiogenic stimuli, endothelial cells can re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels which are functionally adapted to their tissue environment. This process of angiogenesis, concurrent with tissue development and regeneration, depends on the tightly controlled balance between positive and negative signals for endothelial cell proliferation, migration, differentiation and survival. Previously identified growth factors promoting angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF-α (for references, see Folkman, 1995; Friesel and Maciag, 1995; Mustonen and Alitalo, 1995). However, VEGF, which was identified ~10 years ago (Senger *et al.*, 1983), has been the only growth factor relatively specific for endothelial cells. Thus the newly identified factors VEGF-B (Olofsson *et al.*, 1996) and VEGF-C (the present data) increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability and perhaps other endothelial functions.

## Materials and methods

### *Cell culture*

Human prostatic adenocarcinoma PC-3 cells (American Type Culture Collection CRL 1435) were cultured in Ham's F12 medium supplemented with 7% fetal calf serum (FCS); 293 EBNA cells (Invitrogen) and NIH 3T3-Flt4 cells (Pajusola *et al.*, 1993) in DMEM–10% FCS; PAE-KDR cells (Waltenberger *et al.*, 1994) in Ham's F12 medium–10% FCS, BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). After reaching confluence the monolayers of PC-3 cells were cultured for 5 days in Ham's F12 medium without FCS. CM was then collected, clarified by centrifugation at 10 000 *g* and used for purification of VEGF-C.

### *Analysis of stimulation of the receptors*

Confluent NIH 3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 media, respectively, supplemented with 0.2% BSA and then incubated for 5 min with the analysed media. Recombinant human VEGF (R&D Systems) and PDGF-BB were used as control stimulating agents. The cells were washed twice with ice-cold Tris-buffered saline (TBS) containing 100 μM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16 000 *g* for 20 min and incubated for 3–6 h on ice with 3–5 μl of antisera specific for Flt4 (Pajusola *et al.*, 1993), KDR or PDGFR-β (Claesson-Welsh *et al.*, 1989; Waltenberger *et al.*, 1994). Recombinant human PDGF-BB as well as antisera specific for KDR and PDGFR-β were kindly provided by Dr Lena Claesson-Welsh. Immunoprecipitates were bound to protein A–Sepharose, washed three times with TBS containing 1 mM PMSF and 1 mM sodium orthovanadate, twice with 10 mM Tris–HCl pH 7.4 and subjected to SDS–PAGE in a 7% gel (Laemmli, 1970). Polypeptides were transferred to nitrocellulose by Western blotting and analysed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and the ECL method (Amersham).

### *Production and purification of baculoviral Flt4EC protein*

The segment of Flt4 cDNA (GenBank Accession Number X68203) encoding EC was amplified in PCR using primers which encoded six additional C-terminal His residues followed by a stop codon, and added *Bam*HI sites at both ends. The amplified fragment was then cloned into the *Bam*HI site in the pVTBac plasmid (Tessier *et al.*, 1991), which was used to generate a Flt4EC baculovirus. The Flt4EC protein was purified from the culture medium of baculovirus-infected High-Five cells (Invitrogen) by Ni-NTA affinity chromatography (Qiagen) and coupled to CNBr-activated Sepharose 4B (Pharmacia; 5 mg of Flt4 EC/ml Sepharose resin).

### *Isolation and N-terminal sequence analysis of VEGF-C*

Eight litres of PC-3 CM was concentrated 80-fold using a 10 kDa cut-off ultrafiltration membrane (Filtron Technology Corporation) and incubated with the recombinant Flt4EC–Sepharose affinity matrix. The affinity matrix was washed successively with PBS and 10 mM PB (pH 6.8) and the bound material was eluted step-wise with 100 mM glycine–HCl, successive eluates having pHs of 4.0, 2.4 and 1.9. Eluates were collected in tubes containing 1/4 volume of 1 M Na-phosphate pH 8.0, dialysed against 1 mM Tris–HCl pH 7.5 and the aliquots were analysed for their ability to stimulate tyrosine phosphorylation of VEGFR-3.

Two fractions eluted from the affinity matrix at pH 2.4 were combined, vacuum dried and subjected to SDS–PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immibilon-P transfer membrane (Millipore) and visualized by staining of the blot with Coomassie blue R-250. The region containing only the stained 23 kDa band was cut from the blot and subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems).

### *Cloning of VEGF-C from a PC-3 cell expression library*

Six micrograms of poly(A)$^+$ RNA derived from PC-3 cells was used to prepare an oligo(dT)-primed cDNA library using the Librarian kit of Invitrogen. The first PCR was carried out using 1 μg of DNA from the library and the primers marked in the Figure 3A: 1. 5′-GCAGARG-ARACNATHAA-3′ (wherein R is A or G, N is A, G, C or T and H is A, C or T) and 2. 5′-GCAYTTNARDATYTCNGT-3′ (wherein Y is C or T and D is A, G or T). Two successive PCRs were carried out using 1 U per reaction of DynaZyme (Finnzymes), at an extension temperature of 72°C for 43 cycles, the first three cycles at annealing temperature 33°C for 2 min and the remaining ones at 42°C for 1 min. A band of the expected size (57 bp) was re-amplified for 30 cycles in the latter conditions, cloned into a pCRII vector (Invitrogen) and sequenced. All six clones analysed contained the sequence encoding the expected N-terminal peptide (although they were later found also to have mismatches with the final sequence of the cloned cDNA). Based on the unique nucleotide sequence obtained two pairs of nested primers were designed to amplify the complete 5′-end of the cDNA. The primers were 3. 5′-TAATACGACTCACTATAGGG-3′ and 4. 5′-TCNGTGTTGTAGTGTG-CTG-3′, the former corresponding to the pcDNA1 vector used for construction of the library. 'Touchdown' PCR was used (Don *et al.*, 1991). The annealing temperature of the two first cycles was 62°C and subsequently 1°C less in steps of two cycles until a final temperature of 53°C was reached, at which temperature 16 additional cycles were carried out. Annealing time was 1 min and extension at 72°C for 1 min. The products of the first amplification (1 μl of a 1:100 dilution in water) were used in the second amplification reaction employing the nested

primers 5, 5'-TCACTATAGGGAGACCCAAGC-3' and 6, 5'-GTTCGTA-GTGTGCTGCAGCGAATTT-3'. The annealing temperature was decreased in Touchdown PCR from 72°C to 66°C and continued with 18 additional cycles at 66°C. The annealing time was 1 min and extension at 72°C for 2 min. A product of ~220 bp was cloned into the pCR II vector, sequenced and found to contain the 5'-end of the VEGFR-3 ligand cDNA. This fragment was digested with *Eco*RI, and the resulting 153 bp fragment was labelled with [$^{32}$P]dCTP and used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

### *Expression and detection of recombinant VEGF-C*

The 2.0 kb insert of the VEGF-C clone in pcDNA1 vector was cut out from the vector using *Hin*dIII and *Not*I restriction enzymes and ligated into the corresponding sites in the pREP7 expression vector (Invitrogen). The resulting plasmid was transfected into 293 EBNA cells using a calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. The culture medium was changed to DMEM–0.2% BSA 48–72 h after transfection and after an additional 24 h this medium was collected, clarified by centrifugation and used for studies of the effects of VEGF-C. In some cases CM was concentrated using Centriprep-10 devices (Amicon).

Metabolic labelling of 293 EBNA cells transfected with the VEGF-C construct was carried out by addition of 100 μCi/ml of Pro-mix™ L-[$^{35}$S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 2 h the cell layers were washed twice with PBS and the medium was then replaced with DMEM–0.2% BSA. After 1, 3, 6, 12 and 24 h of subsequent incubation the culture medium was collected, clarified by centrifugation, concentrated and VEGF-C was bound to 30 μl of a slur of Flt4EC–Sepharose overnight at 4°C, followed by three washes in PBS, two washes in 20 mM Tris–HCl pH 7.5, alkylation, SDS–PAGE and autoradiography.

### *Endothelial cell migration in three-dimensional collagen gel*

The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2× MEM and two volumes of MEM containing 10% newborn calf serum (NCS) to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with ~1 mm thick layer of the solution, which was allowed to polymerize at 37°C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 min the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% NCS) solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, ME) were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm and the media were daily pipetted into the wells. Photomicrographs of the cells migrating out from the spot edge were taken after 6 days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimide (1 μg/ml, Hoechst 33258, Sigma).

## Acknowledgements

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# Proteolytic processing regulates receptor specificity and activity of VEGF-C

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**The recently identified vascular endothelial growth factor C (VEGF-C) belongs to the platelet-derived growth factor (PDGF)/VEGF family of growth factors and is a ligand for the endothelial-specific receptor tyrosine kinases VEGFR-3 and VEGFR-2. The VEGF homology domain spans only about one-third of the cysteine-rich VEGF-C precursor. Here we have analysed the role of post-translational processing in VEGF-C secretion and function, as well as the structure of the mature VEGF-C. The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2. Recombinant 'mature' VEGF-C made in yeast bound VEGFR-3 ($K_D$ = 135 pM) and VEGFR-2 ($K_D$ = 410 pM) and activated these receptors. Like VEGF, mature VEGF-C increased vascular permeability, as well as the migration and proliferation of endothelial cells. Unlike other members of the PDGF/VEGF family, mature VEGF-C formed mostly non-covalent homodimers. These data implicate proteolytic processing as a regulator of VEGF-C activity, and reveal novel structure–function relationships in the PDGF/VEGF family.**

*Keywords*: angiogenesis/growth factor/proteolytic processing/VEGF/VEGF-C

## Introduction

Angiogenesis, the formation of blood vessels by sprouting from pre-existing ones, is regulated by a balance between positive and negative regulators (Hanahan and Folkman, 1996). Vascular endothelial growth factor (VEGF) belongs to the platelet-derived growth factor (PDGF)/VEGF family and is a major inducer of angiogenesis in normal and pathological conditions (Dvorak *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Ferrara, 1997). The biological effects of VEGF are largely specific for endothelial cells and include stimulation of their proliferation, migration and tube formation, and regulation of vascular permeability (Dvorak *et al.*, 1995; Klagsbrun and D'Amore, 1996; Ferrara, 1997). Another growth factor of the VEGF family, placenta growth factor (PlGF), is expressed predominantly in the placenta; it has minimal angiogenic activity, but is able to heterodimerize with and to modulate the effects of VEGF (Maglione *et al.*, 1991; Park *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996).

VEGF binds to and induces biological responses via two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR), expressed mainly in endothelial cells (see Mustonen and Alitalo, 1995; Shibuya, 1995 for references). PlGF is exclusively a ligand for VEGFR-1 (Park *et al.*, 1994). VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig)-like loops in the extracellular domain (EC), a single transmembrane region and a tyrosine kinase domain, interrupted by an insert of 60–70 amno acid residues (de Vries *et al.*, 1992; Terman *et al.*, 1992; Shibuya, 1995).

Three novel growth factors strikingly similar to VEGF and PlGF have been identified recently. These factors are the VEGF-B/VEGF-related factor (VRF) (Grimmond *et al.*, 1996; Olofsson *et al.*, 1996a), VEGF-C/VEGF-related protein (VRP) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and c-*fos*-induced growth factor (FIGF) (Orlandini *et al.*, 1996). VEGF-B is most closely related to VEGF and is able to form heterodimers with it (Olofsson *et al.*, 1996a,b). VEGF-C and FIGF are similar in that both have N- and C-terminal extensions flanking a VEGF homology domain. Their C-terminal propeptides contain tandemly repeated motifs with a spacing of cysteine residues typical of Balbiani ring 3 protein (BR3P) (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Lee *et al.*, 1996; Orlandini *et al.*, 1996). Thus, VEGF-C and FIGF comprise a novel subgroup of the PDGF/VEGF family.

The receptors for VEGF-B and FIGF have not yet been identified, while VEGF-C is a ligand for two receptors, VEGFR-3 (Flt4) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and VEGFR-2 (Joukov *et al.*, 1996). VEGFR-3 differs from the two other VEGFRs by being proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Aprelikova *et al.*, 1992; Pajusola *et al.*, 1992, 1993; Galland *et al.*, 1993) and by being expressed in angioblasts of the head mesenchyme and in the veins of embryos, and selectively in lymphatic endothelia thereafter (Kaipainen *et al.*, 1995). The paracrine expression patterns of VEGF-C and VEGFR-3 in many tissues suggest that VEGF-C may function in angiogenesis of the lymphatic vasculature (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). On the other hand, the ability of VEGF-C to activate VEGFR-2 points to its possible functional redundancy with VEGF.

The VEGF-C precursor is more than twice as large as the mature polypeptide, initially isolated from PC-3 cell culture media (Joukov *et al.*, 1996). This, combined with the unusual structure of the precursor, raised questions

about the role of its proteolytic processing, possibly affecting receptor specificity, affinity and biological activity. These questions have been addressed in the present study.

## Results

### *Characterization of VEGF-C antibodies and mapping of peptide epitopes in reduced and alkylated VEGF-C polypeptides*

To study VEGF-C processing, we first generated antisera recognizing two different regions of the VEGF-C precursor. Antiserum 882 was obtained by immunization with a synthetic peptide corresponding to amino acid residues 2–18 of the N-terminus of mature secreted human VEGF-C [residues 104–120 of the VEGF-C prepropeptide (Joukov *et al.*, 1996); EMBL, GenBank and DDBJ entry X94216]. Antiserum 905 was raised against the N-terminus of the putative VEGF-C propeptide (residues 33–54) (see Figure 3). These antisera and the extracellular domain of VEGFR-3 (R-3EC) were then compared for their ability to bind metabolically labelled recombinant VEGF-C from the conditioned media (CM) of transfected 293-EBNA cells. Both antibodies precipitated VEGF-C forms with molecular masses of 15, 21, as well as a doublet of 29/31 kDa (Figure 1A, lanes 3 and 5, arrows). At higher levels of VEGF-C expression, polypeptides of 43 and 58 kDa were also detected in the immunoprecipitates (Figures 1B and 2). Importantly, both antibodies immunoprecipitated the VEGF-C forms which were able to bind VEGFR-3 (Figure 1A, lane 2). The doublet of 29/31 kDa was the major component of the immunoprecipitates. The 21 kDa band was precipitated by antiserum 905 less efficiently than by antiserum 882, suggesting that a fraction of this form is bound to (a) polypeptide(s) containing also the N-terminal VEGF-C sequence recognized by antiserum 905. Pre-treatment of the antisera with the corresponding peptides used for immunizations abolished their ability to immunoprecipitate the above-mentioned polypeptides (Figure 1A, lanes 4 and 6), indicating that they were specific for VEGF-C.

In order to explore the structure of the VEGF-C peptides further, we compared the abilities of the antisera to bind VEGF-C after reduction and alkylation of disulfide bonds. This treatment prevented the precipitation of the 29 and 43 kDa polypeptides by both antisera and of the 21 kDa form by antiserum 905 (Figure 1B, lanes 1–4). Reduction and alkylation slowed down the migration of the VEGF-C polypeptides in SDS–PAGE, presumably by dissociating intrachain bonds. Therefore, the absence of the 29 kDa form in these conditions could have been due to its co-migration with the 31 kDa component of the doublet. To show that this is not the case, we generated an artificial *N*-glycosylation site in the N-terminal part of VEGF-C by replacing Arg102 with a serine residue, resulting in the NSS(102) peptide (see Figure 3). This mutation slowed down the mobility of the polypeptide normally migrating at 31 kDa and therefore improved the separation of the doublet, thus confirming the above conclusion (data not shown). The mobilities of the 58 and 15 kDa forms were also reduced to 64 and 21 kDa respectively, indicating that these VEGF-C polypeptides contained the appropriate N-terminal peptide of VEGF-C (data not shown). On the other hand, the 21, 29 and 43 kDa forms were not affected by the R102S mutation, suggesting that these polypeptides contain peptide sequences located C-terminally of R102. The specificity of antiserum 905 was demonstrated further by its inability to immunoprecipitate a VEGF-C mutant in which the N-terminal propeptide (residues 32–102) was deleted (ΔN, see Figures 1B and 3). The ΔN polypeptide, immunoprecipitated with the 882 antiserum, migrated in SDS–PAGE with a mobility corresponding to the size of the deletion (~8 kDa) and it was co-precipitated with an equal amount of another pair of polypeptides of 29–32 kDa, which were not recognized by antiserum 882 upon reduction/alkylation of disulfide bonds. These polypeptides were considered to represent heterogenously cleaved/glycosylated C-terminal fragments of the ΔN precursor.

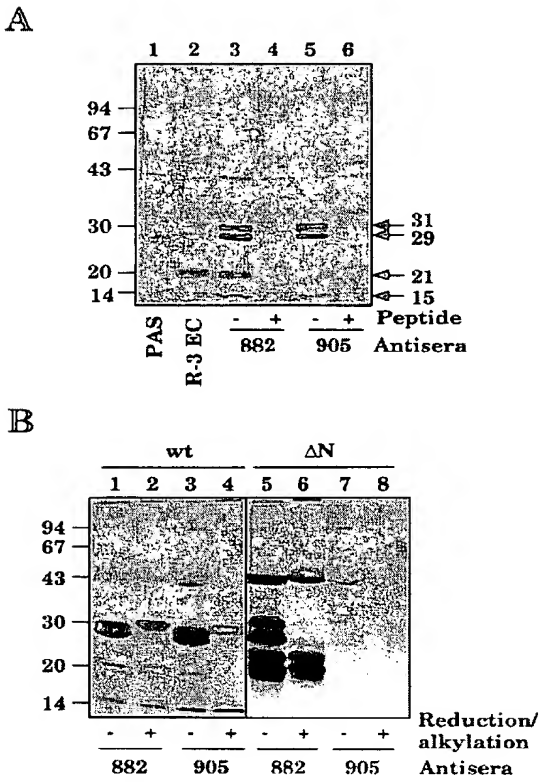


**Fig. 1.** Recognition of VEGF-C polypeptides by antibodies and VEGFR-3. 293-EBNA cells were transfected with VEGF-C, metabolically labelled, and secreted polypeptides were isolated from the medium with subsequent analysis by SDS–PAGE and autoradiography. (**A**) Wild-type VEGF-C was precipitated from CM using protein A–Sepharose (PAS) only (lane 1), PAS and R-3EC (lane 2), antiserum 882 (lanes 3 and 4) or antiserum 905 (lanes 5 and 6). Lanes 4 and 6 show immunoprecipitation using the antisera pre-treated with the corresponding peptides used for immunizations. R-3EC means recombinant soluble extracellular domain of VEGFR-3. (**B**) The antisera 882 and 905 were used to immunoprecipitate wt (lanes 1–4) or ΔN VEGF-C (lanes 5–8) from non-treated CM (lanes 1, 3, 5 and 7) or from CM treated with dithiothreitol and iodoacetamide to reduce and alkylate disulfide bonds (lanes 2, 4, 6 and 8).

### *Biosynthesis, dimerization and proteolytic processing of VEGF-C*

To analyse the kinetics of VEGF-C biosynthesis and processing, we performed metabolic pulse–chase labelling

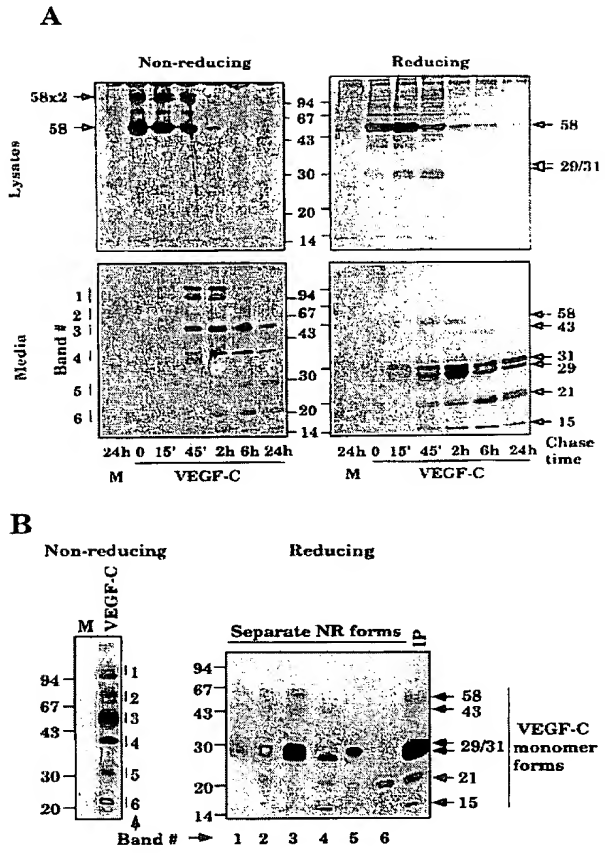


**Fig. 2.** Biosynthesis, dimerization and proteolytic processing of VEGF-C polypeptides. (**A**) Cells were metabolically labelled for 30 min and then chased in non-radioactive medium for the indicated periods of time. The media and cell lysates were immunoprecipitated with antiserum 882 and analysed by SDS–PAGE in 15% gel in non-reducing and reducing conditions. Mock-transfected cultures (M) were analysed after a 24 h chase period. Numbers on the right and on the upper left panels indicate molecular masses (kDa) of the VEGF-C forms. Band numbers of the lower left panel correspond to those in (B). (**B**) Labelled wt VEGF-C polypeptides were first separated in a non-reducing gel (left panel), excised and subjected to SDS–PAGE in reducing conditions (right panel). The corresponding band and lane numbers are indicated.

experiments with cells expressing recombinant VEGF-C. Analysis of the immunoprecipitated VEGF-C polypeptides after different chase periods in non-reducing and reducing conditions revealed that VEGF-C is first synthesized as a 58 kDa precursor, most of which undergoes dimerization before secretion into the culture medium (Figure 2A, upper panels, arrows '58' and '58×2' in lanes 0–45'). It is cleaved further, forming a 29 and a 31 kDa polypeptide (lanes 0–2 h, arrows 29/31), and rapidly secreted, as only a trace amount of the labelled protein was found intracellularly after a 2–6 h chase period. Most of the secreted VEGF-C was made of disulfide-linked low molecular weight forms at all time points analysed (Figure 2A, lower panels), indicating that proteolytic processing accompanies the secretion of VEGF-C. Proteolytic cleavage was detected in cell lysates at 0 min and in the media after a 15 min chase period, but the resulting chains of 31 and 29 kDa were held together by disulfide bonding (compare lanes 15'–2 h run in reducing and non-reducing conditions). At later chase times, these complexes were cleaved further, with concominant accumulation of a 15 and a 21 kDa polypeptide in reducing conditions (lanes 2–24 h). Importantly, this step of the processing occurs after secretion, as no 15 or 21 kDa forms were detected in the cell lysates (upper panels).

To analyse the composition of the different secreted VEGF-C forms we separated $^{35}$S-labelled recombinant VEGF-C polypeptides by SDS–PAGE in non-reducing conditions, excised the polypeptide bands from the gel, reduced the disulfide bonds by treatment of the gel pieces with β-mercaptoethanol and re-analysed the polypeptides in reducing conditions (Figure 2B). The major part of the high molecular weight VEGF-C forms (bands 1–3) gave rise to 29/31 kDa doublets, confirming that the cleaved VEGF-C polypeptides are disulfide-bonded. Only a small fraction of the precursor protein is non-processed or partially processed (products of 58 and 43 kDa in the right hand panel). The low molecular weight components (lanes 4 and 5) contain heterodimerized 15, 21, 29 and 31 kDa polypeptides as well as homodimers of the 31 kDa polypeptide. Interestingly, the monomeric 21 kDa form was also detected (lane 6). The 15 kDa product was disulfide bonded only with the 29 kDa polypeptide (lane 4).

### *Identification of the proteolytically processed and disulfide-linked forms of VEGF-C*

We next used the purified IgG fraction of antiserum 882 to isolate recombinant VEGF-C by affinity chromatography as described in Materials and methods. The purified material contained major polypeptides of 15, 21, 29–30 and 31–32 kDa (data not shown). These polypeptides were subjected to N-terminal amino acid sequence analysis, which gave the sequence $NH_2$-F(32)ESGLDLSDA-COOH for the 15 and 31–32 kDa polypeptides and the sequence $NH_2$-A(112)HYNTEILKS-COOH for the 21 kDa form. Because of our inability to obtain an N-terminal sequence for the 29–30 kDa polypeptide, we generated a VEGF-C construct, containing an N-terminal 6×His tag after the signal sequence (see Figure 3, N-His). Polypeptide components of 32 and 29 kDa were obtained after expression and affinity purification of N-His; analysis of the latter polypeptide revealed the N-terminal amino acid sequence $NH_2$-S(228)LPATL-COOH.

Comparison of the obtained sequences with the sequence of the VEGF-C precursor indicated that polypeptides of 15 and 31 kDa correspond to the N-terminal region of the secreted VEGF-C after cleavage of the signal peptide between Ala31 and Phe32 (Figure 3, arrowhead on the left). The 29 kDa form then represents the C-terminal half of the VEGF-C precursor generated by cleavage between Arg227 and Ser228 (arrowhead on the right). This polypeptide contains one putative *N*-linked glycosylation site and may be cleaved additionally at its C-terminus, as we could not isolate VEGF-C either by using an antiserum against the C-terminal amino acid residues 372–394 or by using the 6×His tag at its C-terminus (data not shown). The 21 kDa form is generated by cleavage of the VEGF-C precursor between Ala111 and Ala112 (grey arrowhead). This cleavage of the recombinant protein thus occurs nine residues C-terminal of the cleavage site located between Arg102 and Thr103, originally described in cultures of

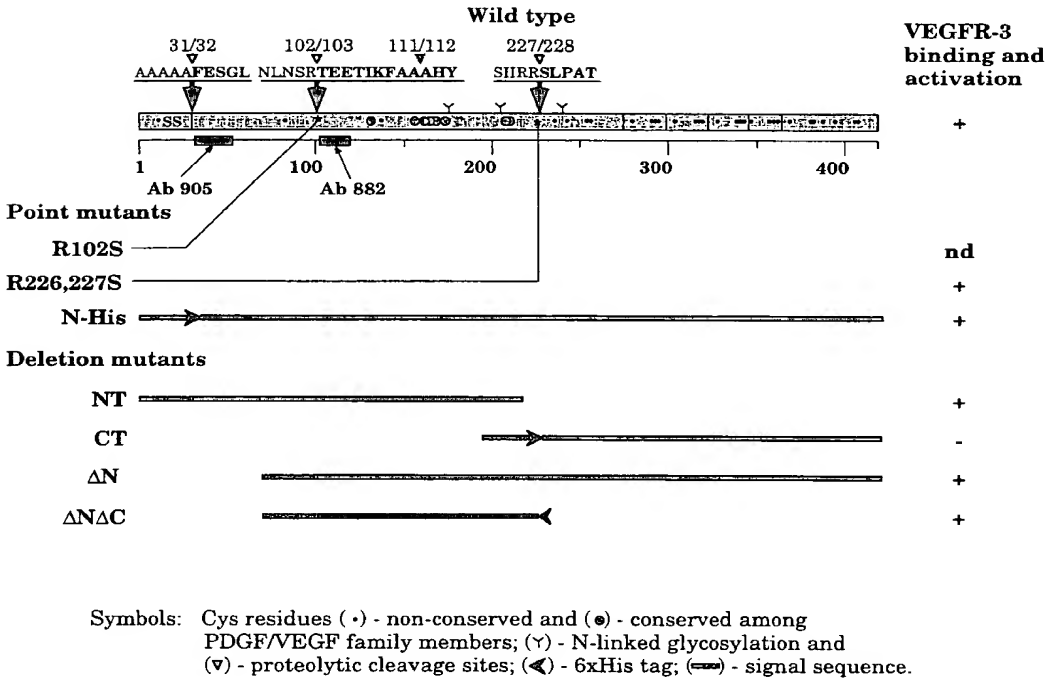


**Fig. 3.** Schematic structure of the wild-type and mutant VEGF-C forms. The VEGF-C prepropeptide is depicted on the top. The signal sequence and the BR3P motifs are outlined. Peptide sequences adjacent to the proteolytic cleavage sites are also shown. Cleavage sites are indicated by arrows, arrowheads and by numbers of the flanking amino acid residues. Peptide sequences revealed by the N-terminal sequence analysis are marked in bold. The diagram under the box shows the scale in amino acid residues. The epitopes recognized by the antibodies 882 and 905 are marked in the diagram as black boxes. The sites of the point mutations are indicated by asterisks. Other VEGF-C mutants are shown as thick black lines, with the signal sequence marked in grey. The ability of the corresponding construct to bind and to activate VEGFR-3 is indicated on the right (nd, not determined).

PC-3 cells (Joukov *et al.*, 1996). Mature polypeptides of 21 and 31 kDa thus contain the entire VEGF homology domain with all eight conserved cysteine residues and two putative *N*-glycosylation sites.

Taking into account these results and analysis of VEGF-C composition in reducing and non-reducing conditions, one can conclude that the main fraction of processed VEGF-C consists of disulfide-bonded N- and C-terminal parts of VEGF-C precursor cleaved between Arg227 and Ser228 (bands 1–3 in Figure 2A and B). Migration of the proteins in non-reducing conditions suggests that the proteolytic processing occurs gradually. Band 1 presumably contains tetrameric complexes made of two 29/31 kDa dimers linked by disulfide bonds (Figure 2B). Analysis of band 2 suggests that it contains trimers made of the 29/31 kDa dimer disulfide-bonded with the 21 kDa form. It also includes small amounts of 43 and 58 kDa polypeptides. However, the major fraction of the 29 and 31 kDa polypeptides migrates in SDS–PAGE as a disulfide-bonded heterodimer (band 3, compare with bands 1 and 2), while most of the 21 kDa form migrates as a monomer (band 6). Some non-processed monomeric 58 kDa precursor and partially processed 43 kDa polypeptide are also included in band 3. Band 4 is formed mainly by the C-terminal half of the VEGF-C precursor (29 kDa), linked by disulfide bonds with its N-terminal fragment of 15 kDa, and band 5 contains the monomeric N-terminal half of the precursor and a small fraction of the 15 and 21 kDa forms heterodimerized by disulfide bonds. An identical processing pattern was observed when R102S VEGF-C was analysed to improve separation of the 29 and 31 kDa components of the doublet (data not shown).

### *VEGF-C is processed similarly in different cell types*

To exclude the possibility that the observed VEGF-C processing pattern is cell type specific and/or occurs only in cells expressing extremely high VEGF-C levels, we analysed VEGF-C isolated from different transfected and non-transfected cells using the 882 antiserum. The main form of both endogenous VEGF-C, produced by PC-3 cells or HT1080 cells, and of the recombinant VEGF-C expressed in 293-EBNA, COS-7 and HT1080 cells is a doublet of 29/31 kDa. The 15, 21 and 58 kDa VEGF-C forms produced by PC-3 and 293-EBNA cells also had similar mobilities in SDS–PAGE. The proteolytic processing of the VEGF-C precursor in COS cells was less efficient when compared with other cell types, possibly due to a high level of expression or a species difference (data not shown). Taken together, these results indicate that VEGF-C is processed similarly in different cell types.

We further analysed whether the 21 kDa VEGF-C form could be produced by proteolytic cleavage of the 31 kDa form. Serum-free CM was collected from PC-3 cell cultures after various periods, concentrated and analysed by Western blotting using the antiserum 882. As can be seen from Figure 4A, the 21 kDa form accumulated in the medium during cell culturing. A similar product could not be detected in 293-EBNA cells, because cleavage of VEGF-C in these cells occurs more C-terminally (see

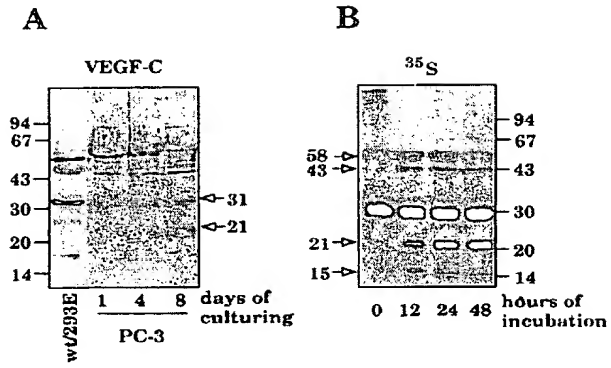


**Fig. 4.** Proteolytic processing of VEGF-C by secreted protease(s). (**A**) PC-3 cells were cultured for the indicated periods in FCS-free medium, which was then concentrated and analysed by Western blotting and detection using the 882 antiserum. Note that the mature VEGF-C 21 kDa form is detected in PC-3 cells, but not in 293-EBNA cells, transfected with VEGF-C (wt/293E). (**B**) CM from 293-EBNA cells, containing labelled VEGF-C, was incubated with concentrated CM from PC-3 cells for the indicated periods of time, and subjected to immunoprecipitation with antiserum 882. The precipitated material was analysed by SDS–PAGE and autoradiography. Note the accumulation of the mature 21 kDa form during incubation.

Figure 3) and thus deletes about half of the peptide sequence recognized by the antiserum. However, the remaining epitope appears to be sufficient for immunoprecipitation of the 21 kDa form using the same antiserum (Figures 1–3 and 4B). Addition of concentrated CM from PC-3 cells to medium containing $^{35}$S-labelled recombinant VEGF-C also caused its proteolytic cleavage, with accumulation of the 21, 43 and 15 kDa products (Figure 4B, arrows), indicating that the protease responsible for VEGF-C cleavage is secreted to the medium. We also observed proteolytic cleavage of the recombinant 31 kDa VEGF-C polypeptide accompanied by simultaneous accumulation of the 21 kDa form upon long-term storage of the CM from transfected 293-EBNA cells (data not shown).

### *'Recombinantly processed' VEGF-C binds VEGFR-3 and VEGFR-2 with high affinity and induces receptor autophosphorylation*

In order to identify and analyse biologically active VEGF-C polypeptides, we generated a panel of deletion mutants of VEGF-C based on the proteolytic processing sites (Figure 3). We found that the ability to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 depends on the presence of the VEGF homology domain. This conclusion is based on the activating properties of polypeptides encoded by the constructs VEGF-C wt, N-His, NT, ΔN, and ΔNΔC, schematically presented in Figure 3 (data not shown). The construct CT, in which the signal sequence was fused to Ser228 of the C-terminal cleavage site, was expressed efficiently and secreted to the culture medium, but it did not stimulate tyrosine phosphorylation of VEGFR-2 or VEGFR-3 (data not shown). The maximal receptor-stimulating activity corresponded to the 21 kDa form, in which both the N- and C-terminal propeptides were deleted at the proteolytic processing sites or in their close proximity (construct ΔNΔC) (see below).

We next produced the ΔNΔC protein in the *Pichia pastoris* yeast expression system and analysed its ability to bind to and stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 expressed in porcine aortic endothelial (PAE) cells (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). Non-transfected PAE cells did not show significant binding of radioiodinated purified ΔNΔC, while specific high affinity binding sites were detected in PAE/VEGFR-3 and PAE/VEGFR-2 cells (Figure 5). The affinities were 135 and 410 pM, respectively, based on Scatchard analysis of the binding data (Figure 5A and B). VEGF-C and VEGF competed with each other for VEGFR-2 binding, VEGF being more efficient in this respect, indicating that the binding involves overlapping sites of the receptor (Figure 5C and D). ΔNΔC, like VEGF, could also be cross-linked to VEGFR-2 on PAE cells (Figure 5F) and it bound to soluble extracellular domains of VEGFR-2 and VEGFR-3. This binding was eliminated by addition of a 30-fold excess of the non-labelled recombinant factor (data not shown). However, ΔNΔC bound neither to the VEGFR-1 extracellular domain (data not shown), nor to the VEGFR-1 expressed in PAE cells (Figure 5E).

Recombinant ΔNΔC, produced both by mammalian and yeast cells, stimulated tyrosine phosphorylation of VEGFR-3 and VEGFR-2 in a dose-dependent fashion at concentrations of 0.2–20 nM (Figure 5G and H and data not shown). This effect was not affected by the presence of the 6×His tag (data not shown). The stimulation of VEGFR-2 was comparable with that of similar concentrations of VEGF. Heparin at 1 μg/ml either did not affect or even decreased binding of ΔNΔC by both receptors (data not shown). Altogether, these data indicate that the proteolytically processed 21 kDa VEGF-C is a ligand and an activator of both VEGFR-3 and VEGFR-2.

### *Mature VEGF-C has VEGF-like activities*

The ability of ΔNΔC to activate VEGFR-2 raised the question of whether it can also induce biological responses characteristic of VEGF. We found that ΔNΔC stimulated the proliferation of bovine capillary endothelial (BCE) cells, although equal stimulation required ~50-fold higher concentrations of VEGF-C in comparison with VEGF (Figure 6A). ΔNΔC, like wt VEGF-C, stimulated the migration of BCE cells in collagen gel, again at higher concentrations when compared with VEGF (Figure 6B). Also, pure recombinant ΔVEGF-C injected subcutaneously into guinea pig skin increased the permeability of blood vessels in a dose-dependent manner (Figure 7A). In this assay, only 4- to 5-fold higher concentrations of ΔNΔC were required compared with VEGF (Figure 7B). Altogether, these data indicate that the proteolytic processing of the VEGF-C precursor generates a biologically active factor which possesses VEGF-like effects on endothelial cells, stimulating their proliferation and migration, as well as the permeability of blood vessels *in vivo*.

### *Proteolytic maturation affects receptor specificity and activity of VEGF-C*

We next addressed the question of whether proteolytic processing affects the ability of VEGF-C to bind and to activate VEGFR-3 and VEGFR-2. In addition to the above-described ΔNΔC, we also generated the VEGF-C R226,227S form in which Arg226 and Arg227, adjacent to the cleavage site, were replaced with serine residues

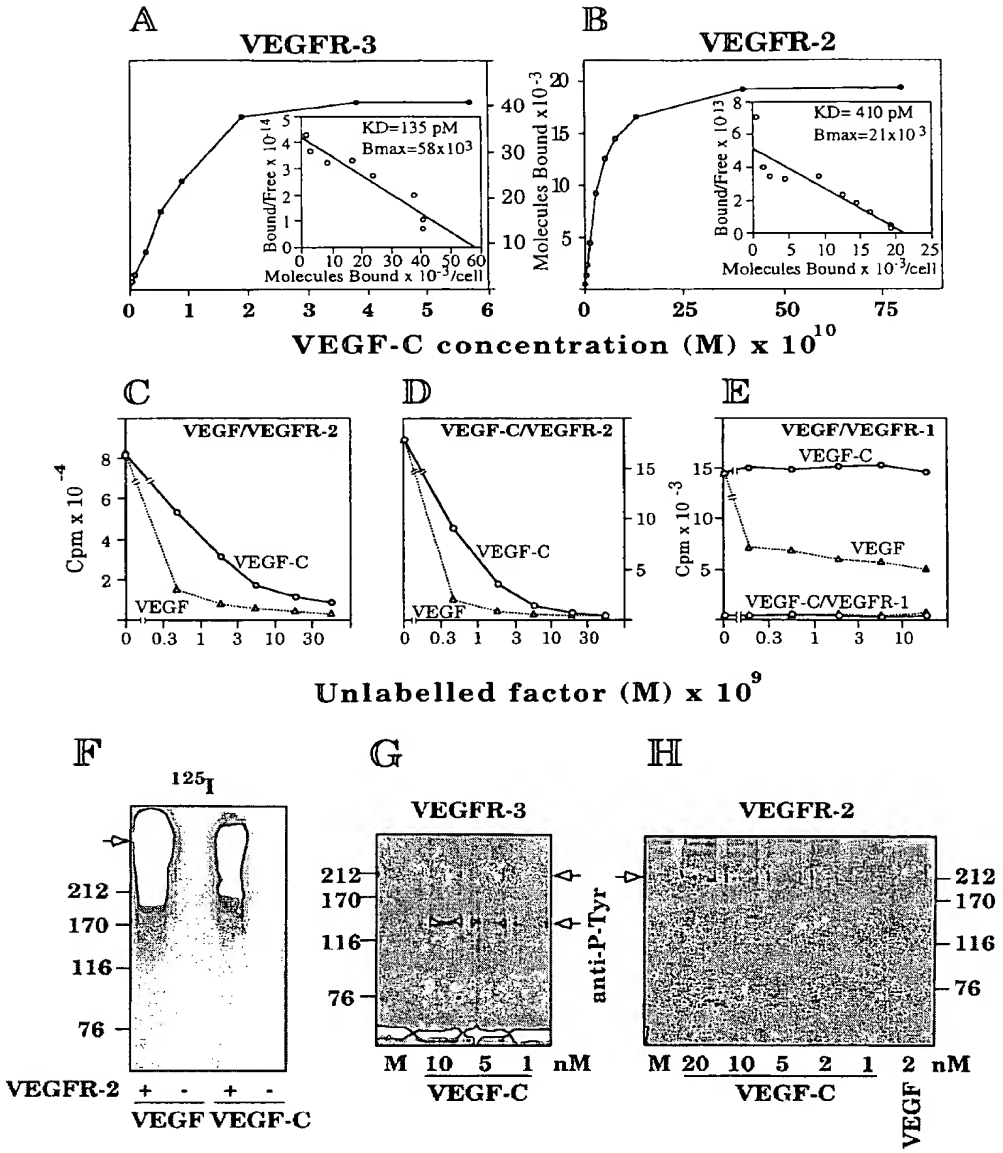


**Fig. 5.** VEGF-C binds to and activates VEGFR-3 and VEGFR-2. (**A**) and (**B**) Saturation binding curves and Scatchard analysis (inserts) of [$^{125}$I]ΔNΔC binding to PAE/VEGFR-3 cells (A) and PAE/VEGFR-2 cells (B). (**C**) and (**D**) Displacement of [$^{125}$I]VEGF (C) or [$^{125}$I]ΔNΔC (D) from VEGFR-2 by VEGF (triangles) and VEGF-C (circles). (**E**) Displacement of [$^{125}$I]VEGF (closed symbols) and [$^{125}$I]ΔNΔC (open symbols) from PAE/VEGFR-1 cells by VEGF (triangles) and ΔNΔC (circles). (**F**) Autoradiogram of VEGFR-2 immunoprecipitates from PAE (–) and PAE/VEGFR-2 (+) cells after cross-linking with [$^{125}$I]VEGF or [$^{125}$I]ΔNΔC. The arrow shows the mobility of the major labelled ligand–receptor complex. (**G**) and (**H**) Stimulation of tyrosine phosphorylation of VEGFR-3 and VEGFR-2 by ΔNΔC at different concentrations. Control lanes show analysis of mock-stimulated cells and treatment with 2 nM VEGF. The tyrosine-phosphorylated receptors are marked by arrows. Note the concentration-dependent phosphorylation of VEGFR-2, and of unprocessed 195 kDa and proteolytically processed 125 kDa VEGFR-3 forms in cells treated with ΔNΔC.

(Figure 3). As a consequence, the proteolytic processing at this site was almost completely abolished, as detected by Western blotting using the 882 antiserum (Figure 8A, lane 2). Small amounts of the 31 and 21 kDa polypeptides were, however, found in [$^{35}$S]R226,227S immunoprecipitates, possibly due to clevage at an alternative site (Figure 9B, lane 1). R226,227S can thus be considered an analogue of the VEGF-C precursor, while wt VEGF-C consists mostly of partially processed 29 and 31 kDa forms, ΔNΔC being an analogue of fully processed, mature VEGF-C (Figure 8A, lanes 3 and 4).

As can be seen from Figure 8B, all processed VEGF-C forms bind to R-3EC, with preferential binding of the 21 kDa form (lanes 2, 5, 7 and 13). Even more striking was the selective binding of the mature 21 kDa form of wt VEGF-C and of ΔNΔC by the VEGFR-2 extracellular domain–alkaline phosphatase fusion protein (R-2EC, lanes 3, 6, 8 and 14). Neither protein A–Sepharose (see Figure

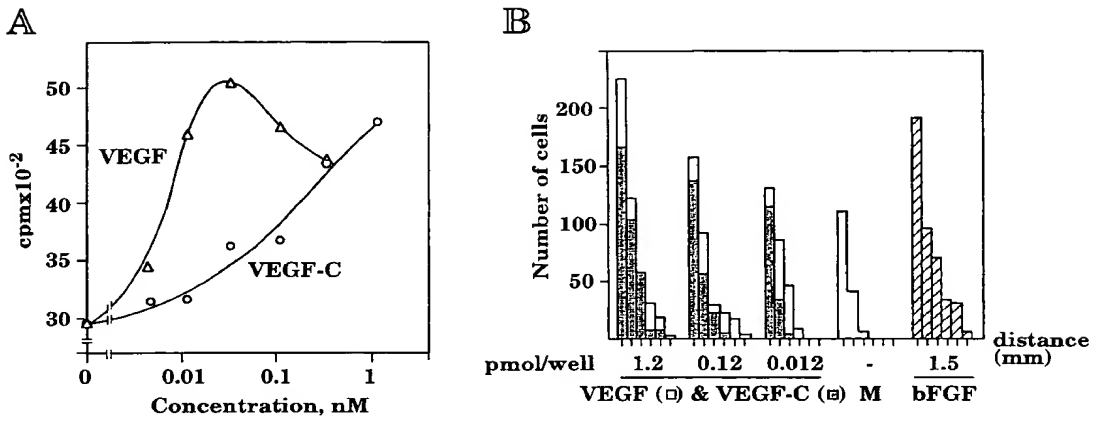


**Fig. 6.** VEGF-like activity of VEGF-C. (**A**) ΔNΔC stimulation of [$^3$H]thymidine incorporation into DNA of BCE cells. Results of one experiment using different concentrations of the factors are shown. Standard deviations were <10%. (**B**) Migration of BCE cells in collagen gel. The diagram shows the number of cells migrating to six different distances (0.6 μm step) starting from the left (marked by vertical ticks). Analysis employed mock medium (M, black bars) or medium with the indicated amounts of VEGF (open bars), ΔNΔC (grey bars) or bFGF (striped bars).

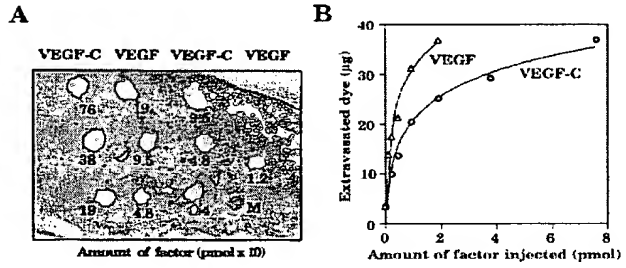


**Fig. 7.** Comparison of VEGF and VEGF-C in the Miles assay for vascular permeability *in vivo*. (**A**) The indicated amounts of VEGF and VEGF-C were injected intradermally to the back region of a guinea pig. The photograph shown was taken 20 min after the injections. Injection of diluent (PBS/BSA) is marked as M. (**B**) Graphs showing the quantitation of the results of the Miles assay as described in Materials and methods.

1, lane 1) nor protein G–Sepharose alone, or in combination with the anti-alkaline phosphatase antibodies (lanes 10 and 11), bound the 21 kDa form, although very small amounts of the the 58 and 31 kDa VEGF-C forms were bound unspecifically (lanes 8, 9 and data not shown). The specificity of VEGF-C binding was supported by the finding that R-2EC bound VEGF, but not PlGF, while R-3EC did not bind VEGF (data not shown).

Analysis of the receptor-bound material in non-reducing conditions revealed that the 60 kDa polypeptide, which was bound preferentially to R-3EC consisted of disulfide-bonded 29 and 31 kDa heterodimers (lane 13, upper arrow). Most of the 21 kDa polypeptide bound to both R-3EC and R-2EC migrated as a monomer in these conditions (lanes 13 and 14, lower arrow). This finding was most surprising with regard to previously published data concerning other VEGF family members (Maglione *et al.*, 1991; Heldin *et al.*, 1993; Olofsson *et al.*, 1996a; Ferrara, 1997).

We next analysed the ability of the described VEGF-C forms to compete with [$^{125}$I]VEGF-C(ΔNΔC) for binding to VEGFR-2 and VEGFR-3. As can be seen from Figure 8C, all VEGF-C mutants displaced [$^{125}$I]VEGF-C from VEGFR-3. The efficiency of displacement was as follows: ΔNΔC>wt>R226,227S, i.e. enhanced binding was obtained upon inclusion of the more mature forms. Recombinant VEGF165 failed to displace VEGF-C from VEGFR-3, but VEGF, ΔNΔC and wt VEGF-C efficiently displaced labelled VEGF-C from VEGFR-2, ΔNΔC being more potent in comparison with wt VEGF-C (Figure 8D). The non-processed R226,227S form showed only weak competition with [$^{125}$I]VEGF-CΔNΔC, which could be attributed either to its much lower affinity for VEGFR-2, or to the presence of a small amount of the mature forms, cleaved at an alternative site (see above).

Next, we studied the ability of the above-mentioned VEGF-C forms to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2. Stimulation of VEGFR-3 and VEGFR-2 autophosphorylation by the different VEGF-C forms in general correlated with their binding properties and with the degree of proteolytic processing (Figure 8E). ΔNΔC showed a higher activity than wt VEGF-C (lanes 3 and 4), and R226,227S had a considerably weaker effect on autophosphorylation of VEGFR-3, and almost no effect on VEGFR-2 autophosphorylation (lane 2). Finally, the ability of different VEGF-C forms to promote vascular permeability was examined in the Miles assay. CM containing the VEGF-C polypeptides were pre-treated with monoclonal anti-VEGF neutralizing antibodies to eliminate the effect of endogenous VEGF produced by 293-EBNA cells. Although the effect of pure VEGF was neutralized in control experiments, the antibody-treated CM still slightly increased vascular permeability, presumably due to the presence of other permeability factors (Figure 8F and data not shown). CM containing wt and ΔNΔC VEGF-C increased vascular permeability, while the effect of R226,227S CM did not differ significantly from that of CM from mock-transfected cells (Figure 8F). Importantly, identical dilutions of CM were used for these experiments and for the experiments presented in Figure 8C–E. A Western blot analysis of CM using anti-VEGF-C antiserum 882 is shown in Figure 8A to illustrate the relative amounts of the factors present.

Because antiserum 882 did not recognize the mature wild-type polypeptide produced by 293-EBNA cells on a Western blot (see above), metabolic labelling and immuno-

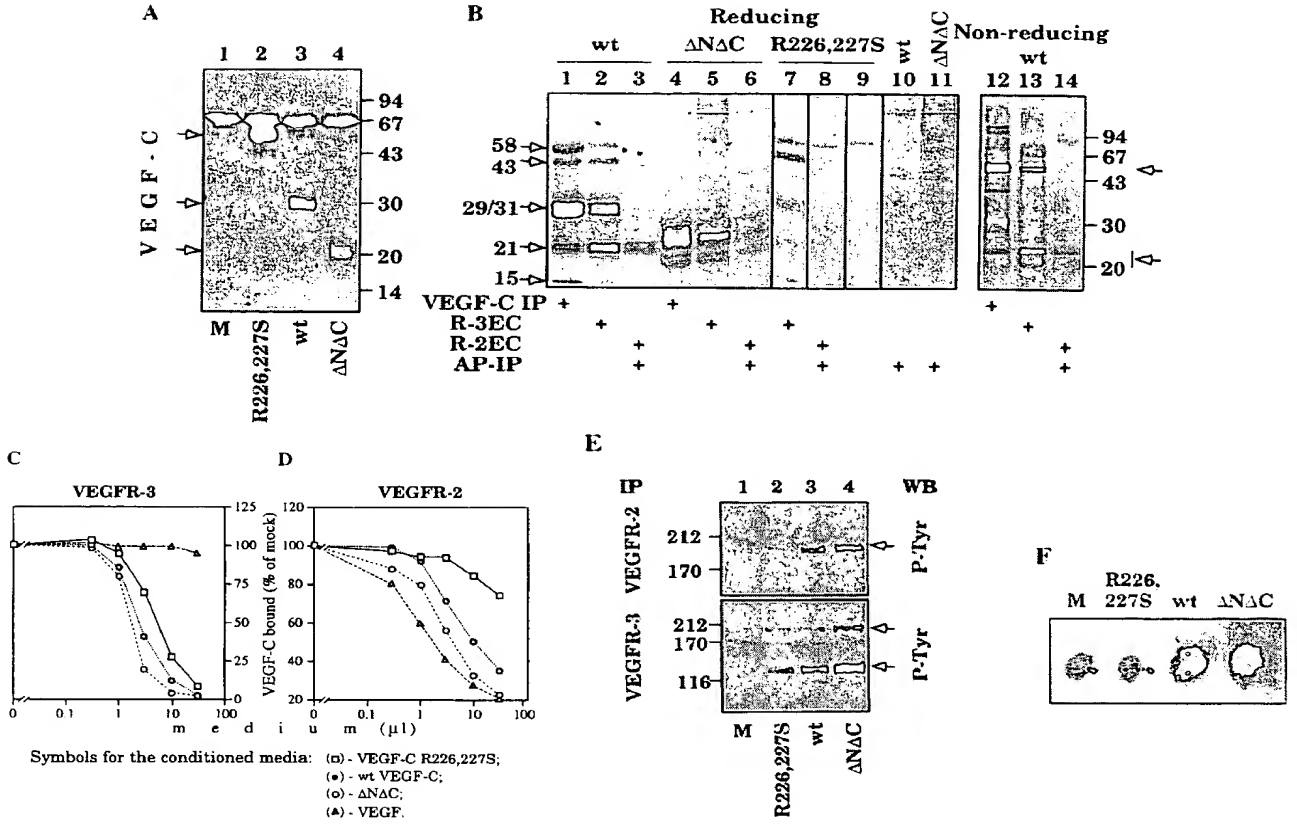


**Fig. 8.** Proteolytic processing increases the ability of VEGF-C to bind to and activate VEGFR-3 and VEGFR-2. (**A**) Detection of three processed VEGF-C forms by antiserum 882 on a Western blot (arrows). VEGF-C concentrations in CM were adjusted by dilutions with medium from mock-transfected cells. The band at 67 kDa is BSA added as a carrier protein. (**B**) Binding of the various VEGF-C forms by the extracellular domains of VEGFR-3 and VEGFR-2. Metabolically labelled VEGF-C from CM of cells transfected with the indicated constructs was bound to R-3EC (lanes 2, 5 and 7) or to R-2EC (lanes 3, 6 and 8). The receptor extracellular domains were precipitated and analysed by SDS–PAGE. The same amounts of CM were immunoprecipitated (lanes 1 and 4) or treated with PAS only (lane 9). Analysis of material in lanes 1–3 in non-reducing conditions is shown in lanes 12–14. Note that the main fraction of the 21 kDa form bound to the extracellular domains of both receptors does not form disulfide-linked dimers (lower arrow), while most of the 29/31 kDa complexes bound to R-3EC are heterodimerized via disulfide bonds (upper arrow). (**C**) and (**D**) Displacement of [$^{125}$I]ΔNΔC from the receptors by the VEGF-C forms. PAE/VEGFR-3 (C) and PAE/VEGFR-2 (D) cells were incubated with trace amounts of [$^{125}$I]ΔNΔC in the absence or presence of different amounts of CM containing the indicated polypeptides, the cells were washed and the amount of bound radioactivity was measured in a γ-counter. Experiments presented in (C), (D) and (E) were carried out using the CM analysed in (A). (**E**) Stimulation of tyrosine phosphorylation of VEGFR-2 (upper panel) and VEGFR-3 (lower panel) by CM from mock-transfected cells (lane 1) and from cells overexpressing the indicated VEGF-C forms. Tyrosine-phosphorylated receptor polypeptides are indicated by arrows. (**F**) Proteolytically processed VEGF-C increases vascular permeability. CM containing ~8 pM of the indicated VEGF-C variants were pre-treated with anti-VEGF neutralizing antibodies and injected intradermally to the back of a guinea pig. The photograph shown was taken 20 min after the injections.

precipitation was carried out to better estimate the relative amounts of each processed form (Figure 9B, lanes 1, 3 and 5). In the experiment presented in Figures 8 and 9, the amount of the 21 kDa polypeptide was approximately one-third of that of the 31 kDa form in the same wt VEGF-C conditioned medium. Taken together, these data indicate that the ability to bind to and to activate VEGFR-3 and VEGFR-2 increases during the proteolytic processing of VEGF-C. Non-processed VEGF-C preferentially binds to and activates VEGFR-3, while the mature 21 kDa VEGF-C form is a high affinity ligand and an activator of both VEGFR-3 and VEGFR-2.

### *Mature form of VEGF-C consists of non-covalent dimers*

Members of the PDGF/VEGF family are active only as dimers. However, as shown above, the proteolytically processed VEGF-C exists mainly as a monomer or a non-disulfide-bonded dimer, which binds VEGFR-3 and VEGFR-2. We were interested in the possibility that dimerization of the processed VEGF-C occurs via non-covalent interactions. Unlike VEGF, which migrates in non-reducing conditions as a dimeric protein of ~44 kDa, most of ΔNΔC migrates as a monomer (Figure 9A). As can be seen from Figure 9B, lanes 6 and 8, about one-half of disuccinimidyl suberate (DSS)-cross-linked VEGF and ΔNΔC migrated as dimers (arrows pointing to lanes 6 and 8 on the right) in reducing conditions. Taking into account that in our conditions ~90% of VEGF migrated as a disulfide-bonded dimer (Figure 9A, lane 1), we conclude that mammalian cells produce ΔNΔC preferentially as a non-covalently bonded dimer (Figure 9A, lane 2 and Figure 9B, lanes 5 and 6). When wt VEGF-C was cross-linked, the amount of the 21 kDa form was

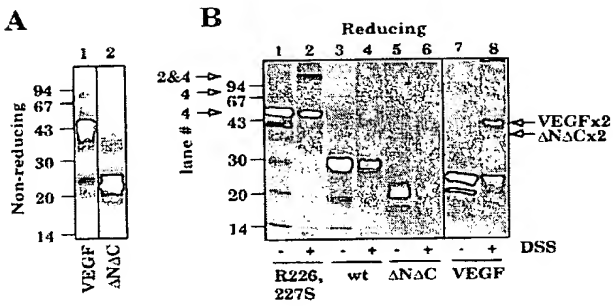


**Fig. 9.** Mature 21 kDa VEGF-C forms non-covalent dimers. (**A**) Metabolically labelled VEGF165 (lane 1) and ΔNΔC (lane 2) immunoprecipitated from CM and analysed by SDS–PAGE in non-reducing conditions. Note that the majority of VEGF migrates as a dimeric protein, while ΔNΔC migrates as a monomer in these conditions. (**B**) Analysis of the VEGF and VEGF-C forms in CM with (+) or without (–) covalent cross-linking using DSS, followed by immunoprecipitation with the antiserum 882 (lanes 1–6) or with VEGF antibodies (lanes 7 and 8). The numbers and arrows point to the cross-linked dimers and multimers detected in the corresponding lanes. Note that approximately equal proportions of ΔNΔC and VEGF migrate as dimers upon cross-linking (arrows on the right). Note also that complexes of ~ 60 and 80 kDa appear upon cross-linking of wt VEGF-C, and complexes of ~120 kDa are formed when R226,227S is cross-linked (arrows on the left).

considerably decreased (Figure 9B, lane 4), suggesting that it is bound to other polypeptides. Also, additional bands of 60, 80 and 120 kDa appeared in reducing conditions (lane 4, arrows on the left). The first of these apparently represents heterodimers of 29 and 31 kDa forms; the 80 kDa complex is most probably a trimer, consisting of 21, 29 and 31 kDa polypeptides, and the 120 kDa band contains two dimerized VEGF-C precursors, most of which are cleaved at the 227R/228S site. When cleavage between Arg227 and Ser228 was abolished (the R226,227S mutant), no cross-linked complexes of 60 and 80 kDa were detected; instead complexes of ~120 kDa were very prominent, both in non-reducing conditions (data not shown) and upon cross-linking (lane 2). These complexes presumably consist of non-processed VEGF-C dimers linked by disulfide bonds. Despite the fact that we were unable to cross-link the complexes completely, these data, along with the analysis of VEGF-C in reducing and non-reducing conditions, clearly show the co-existence of a variety of its di- and multimeric forms, assembled via disulfide bonding and non-covalent interactions. We also found that recombinant N- and C-termini of VEGF-C were able to form heterodimers when co-expressed in mammalian cells (data not shown), emphasizing the existence of a mechanism for the formation of such dimers in mammalian cells.

## Discussion

### *Proteolytic processing of VEGF-C*

Based on the described results, we propose the VEGF-C proteolytic processing model, which is presented schematically in Figure 10. This model resembles the model for the proteolytic processing of PDGF, especially of PDGF-BB (Östman *et al.*, 1988, 1992) in that: (i) the proteolytic cleavages occur after the formation of disulfide-bonded precursor dimers; (ii) both N- and C-terminal propeptides are subject to cleavage; and (iii) a variety of processed forms are secreted. On the other hand, there are several important differences between PDGF-BB and VEGF-C, concerning both their processing and the structure of the mature growth factors.

VEGF-C is released rapidly from cells upon secretion. Upon biosynthesis, two VEGF-C polypeptides, oriented in an anti-parellel fashion, form a dimer linked by disulfide bonds and apparently also by non-covalent bonds. Anti-parallel dimerization is supported by the disulfide bonding of the N- and C- terminal halves (29/31 kDa doublet) of the precursor. Precursor homodimerization is thus followed by the key event in the proteolytic processing—the cleavage between Arg227 and Ser228 dividing the VEGF-C precursor into nearly equal halves. This cleavage site was confirmed by N-terminal peptide sequence analysis and by the R226,227S substitutions, which abolished the cleavage. The N-terminal part of the 31 kDa form contains the VEGF homology domain, and the cysteine-rich C-terminus of the 29 kDa form contains the BR3 motifs. Similarly to PDGF, this processing step occurs in the producer cells, either close to the end of the secretory pathway or at the plasma membrane, because only small amounts of cleaved VEGF-C precursor can be detected in the cell lysates. Most of the secreted VEGF-C is then already cleaved between Arg227 and Ser228, and the resulting polypeptides initially form a tetramer, originating from two precursor polypeptides bound to each other. This processing step is probably conserved in evolution, because the human, mouse and avian VEGF-Cs, as well as FIGF, contain the same amino acid sequence, SIIRRS, surrounding the cleavage site, and a doublet of polypeptides of ~30 kDa is detected in the corresponding immunoprecipitates from the CM of transfected cells (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Orlandini *et al.*, 1996). Morover, most of the VEGF-C secreted by different cell types migrates in reducing conditions as a doublet of 29/31 kDa (data not shown).

The efficient secretion of the R226,227S mutant as well as the presence of small amounts of unprocessed wt VEGF-C precursor in the CM indicate that the intracellular proteolytic cleavage is not a prerequisite for VEGF-C secretion. The C-terminal cleavage of the PDGF-BB precursor occurs in close proximity to the site which corresponds to Arg227 in VEGF-C. Cleavage of high molecular weight VEGF forms by plasmin, with release of diffusible VEGF, also takes place only 10 amino acid residues N-terminal of the VEGF-C cleavage site when VEGF and VEGF-C sequences are aligned (Houck *et al.*, 1992; Keyt *et al.*, 1996a). The proteases responsible for the cleavage of these growth factors might differ, however, because of differences in peptide sequences surrounding the cleavage sites (Östman *et al.*, 1988).

The next step of the proteolytic processing of VEGF-C, which removes the N-terminal propeptide, occurs extracellularly, because the 21 kDa polypeptide was not detected in cell lysates. This differs from the proteolytic processing of PDGF, which occurs exclusively intracellularly (Östman *et al.*, 1992). The 21 kDa form accumulated even upon incubation of cell-free CM, indicating that the cleavage is catalysed by (an) as yet unknown secreted protease(s). Differences in the cleavage sites in cultures of PC-3 and 293-EBNA cells (A111/A112 and R102/

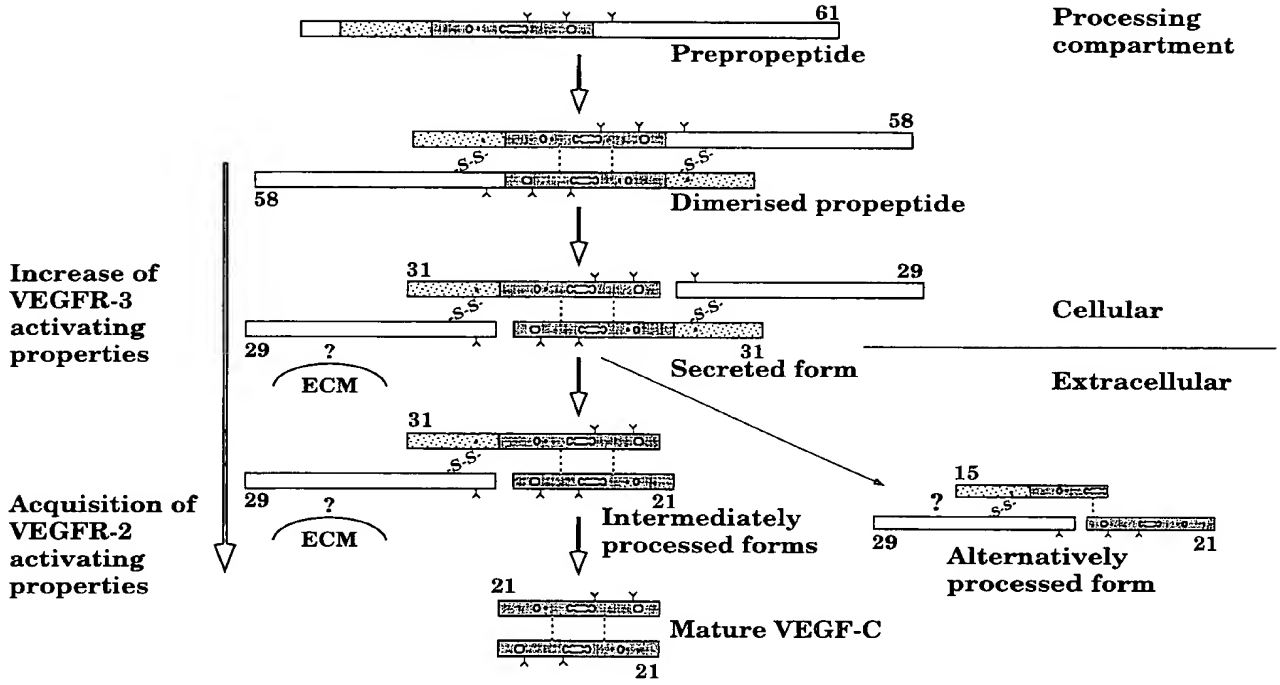


**Fig. 10.** Schematic model of the proteolytic processing of VEGF-C. The regions of VEGF-C polypeptide are marked as follows: signal sequence, black box; VEGF homology domain, grey box; N-terminal and C-terminal propeptides, dotted and open boxes, respectively. Cysteine residues and putative sites of N-linked glycosylation are shown as in Figure 3; the cysteine residues in the C-terminal propeptide are not marked for clarity. Numbers indicate molecular mass (kDa) of the corresponding polypeptide in reducing conditions. Disulfide bonds are marked as -S-S-; non-covalent bonds as dotted lanes. The hypothetical binding of the C-terminal domain to the extracellular matrix (ECM), and the proposed structure of the alternatively processed VEGF-C are indicated with question marks. The proteolytic generation of a small fraction of disulfide-linked 21 kDa forms is not indicated in the figure. Several intermediate forms are also omitted to simplify the scheme.

T103, respectively) suggest that such proteases are either redundant or that cell type-specific factors determine the exact cleavage sites. This stage of the proteolytic processing occurs in a more gradual fashion, and it finally gives rise to mature VEGF-C, composed of two VEGF homology domains bound by non-covalent interactions.

The small amounts of the shortest identified VEGF-C polypeptide of 15 kDa represent the N-terminal part of the precursor, which binds via disulfide bond(s) with the C-terminal 29 kDa propeptide. The mobility of the 15 kDa form in SDS–PAGE and its recognition by both antisera used in the present study suggest that it contains most of the VEGF homology domain, excluding the first glycosylation site, indicating the existence of an additional proteolytic processing site. Interestingly, this form is very similar to the short splicing variant (clone vh 1.1), reported for VRP (Lee *et al.*, 1996), and thus it may have an analogous, so far unknown function. It is possible that the 15 kDa polypeptide interacts with the 21 kDa form, giving rise to a trimer. It might have an antagonistic activity, competing with the mature ligand for receptors. Small amounts of a secreted 43 kDa form were also detected, but we could not isolate enough of this form to determine its peptide sequence. However, the inability of both VEGF-C antisera to precipitate this form upon reduction/alkylation of disulfide bonds and the correlation of its appearence with the appearance of the 15 kDa form suggest that it might represent the complementary C-terminal part of the VEGF-C precursor after cleavage of the 15 kDa N-terminal part.

Several lines of evidence indicate that mature VEGF-C made by transfected overexpressing cells is a non-covalent dimer. Most of the mature VEGF-C and ΔNΔC migrate at 21 kDa in reducing and non-reducing conditions. Despite this, similar proportions of dimeric molecules (~50%) are detected upon cross-linking the recombinantly produced VEGF and ΔNΔC. Of the various forms, these have the highest affinity for VEGFR-3 and VEGFR-2. The tetra- and trimeric VEGF-C molecules, which were detected upon cross-linking, presumably involve both disulfide bonds connecting the N- and C-terminal parts of separate precursor chains and non-covalent interactions between the two VEGF homology domains (see Figure 10). Subsequent removal of the N-terminal propeptide from both precursors would then explain the formation of the non-disulfide-linked mature VEGF-C dimer.

Absense of interchain disulfide bonds is unusual for the members of the PDGF/VEGF family, in which the second and fourth cysteine residues are involved in anti-parallel interchain disulfide bonds (Andersson *et al.*, 1992; Pötgens *et al.*, 1994). These disulfide bonds are crucial for dimerization and biological activity of VEGF, but are not required for dimerization or mitogenic effects of PDGF-BB (Andersson *et al.*, 1992; Kenney *et al.*, 1994; Pötgens *et al.*, 1994). It has also been shown that the dimer interface in PDGF-BB is sufficient to stabilize the dimer substantially in the absence of a covalent linkage (Oefner *et al.*, 1992). It is possible that such an interaction of the mature polypeptide chains is tighter in VEGF-C than in PDGF-BB. Interestingly, the mature VEGF-C contains an extra cysteine residue at position 137, located between

the first and the second cysteine residue characteristic of the PDGF/VEGF family. This residue is also conserved in mouse and avian VEGF-C (Kukk *et al.*, 1996; A.Eichmann *et al.*, unpublished data) and in FIGF (Orlandini *et al.*, 1996). This residue remains unpaired after cleavage of the N-terminal propeptide, which contains another unpaired cysteine residue.

### *Properties of the mature VEGF-C*

The results with ΔNΔC, which mimics mature VEGF-C, support our earlier observations (Joukov *et al.*, 1996; Kukk *et al.*, 1996) and clearly indicate that proteolytically processed VEGF-C binds to and activates both VEGFR-3 and VEGFR-2. A single class of high affinity sites was observed in PAE/VEGFR-3 cells ($K_D$ = 135 pM) and PAE/VEGFR-2 cells ($K_D$ = 410 pM). These values are of similar magnitude to the affinities reported for the VEGF–VEGFR-2 interaction (Terman *et al.*, 1992; Waltenberger *et al.*, 1994). VEGF-C and VEGF displace each other from VEGFR-2, indicating that the same region of this receptor is involved in binding of both ligands. Surprisingly, none of the three basic residues reported to be critical for VEGFR-2 binding by VEGF (Keyt *et al.*, 1996b) are conserved in VEGF-C, indicating that other residues of VEGF-C are important for its interaction with VEGFR-2. VEGF-C also dose-dependently stimulated autophosphorylation of VEGFR-3 and VEGFR-2 but, in agreement with previously reported data (Lee *et al.*, 1996), we could not detect binding of VEGF-C to VEGFR-1.

Like VEGF, VEGF-C stimulates the proliferation and migration of endothelial cells and increases vascular permeability, albeit at concentrations higher than required for VEGF. These activities are probably mediated through VEGFR-2 activation (Park *et al.*, 1994; Waltenberger *et al.*, 1994). Higher effective concentrations of VEGF-C may depend on its lower affinity for VEGFR-2, and on its inability to bind VEGFR-1, precluding the formation of VEGFR-1–VEGFR-2 heterodimers, which may be required for maximal biological responses to VEGF (Waltenberger *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996; Clauss *et al.*, 1996). The role of VEGFR-2 in the effects of VEGF-C *in vivo* remains to be studied.

The paracrine relationship between the VEGF-C and VEGFR-3 expression patterns in embryos suggests that VEGF-C functions in the formation of the venous and lymphatic vascular systems, where VEGFR-3 is expressed (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). Our unpublished observations from transgenic mice support such a notion (Jeltsch *et al.*, 1997). However, the redundancy of VEGF-C with VEGF in VEGFR-2-mediated signalling might account for the interesting observations that VEGF –/– mice have delayed endothelial cell differentiation, while in VEGFR-2 –/– mice both haematopoietic and endothelial cell development is aborted, suggesting that (a) VEGFR-2 ligand(s) distinct from VEGF (such as VEGF-C) might play an important role in these processes (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Indeed, VEGF-C expression is first detected in day 7 p.c. embryos (Kukk *et al.*, 1996), which is striking, considering the first appearance of the VEGFR-3 mRNA on day 8.5 of gestation (Kaipainen *et al.*, 1995). The question of whether VEGF-C is indeed another factor essential for the development of haematopoietic/endothelial cells will need further studies.

### *Significance of the proteolytic processing of VEGF-C*

Our results demonstrate that during proteolytic processing, VEGF-C acquires the ability to bind to and to activate VEGFR-2, and increases its affinity and activating properties towards VEGFR-3. Many other cytokines and growth factors are also synthesized initially as precursors. These include members of the epidermal growth factor (EGF) family, and the transforming growth β (TGF-β) superfamily, interleukins 1α and 1β, nerve growth factor, hepatocyte growth factor (HGF) and others. Proteolytic processing of TGF-β and HGF precursors is an essential step in the formation of the biologically active ligands (Naka *et al.*, 1992; Vigna *et al.*, 1994; Dubois *et al.*, 1995). Our data indicate that the proteolytic processing of VEGF-C plays a similar role, endowing the mature polypeptide with the ability to activate VEGFR-2. Taking into account the presence of VEGFR-2 in many types of endothelia and the broad expression pattern of VEGF-C, we propose that the biosynthesis of VEGF-C as a precursor prevents unnecessary angiogenic effects, elicited via VEGFR-2, and allows VEGF-C to signal preferentially via VEGFR-3, which is restricted to the venous endothelia during early stages of development and to the lymphatic endothelium during later stages. In certain circumstances, proteolytic processing would release mature VEGF-C, which is able to signal via both VEGFR-3 and VEGFR-2. It is also possible that activation of both VEGFR-3 and VEGFR-2, either as homo- or as heterodimers, is necessary to elicit a complete biological response to VEGF-C. In this case, proteolytic processing might provide a regulatory mechanism which gives the possibility of fine tuning the biological functions of VEGF-C. Also, the extracellular processing step introduces an additional level of regulation of the VEGF-C activity.

An important function of the proteolytic processing of PDGF, and possibly also of certain VEGF isoforms, is to control the bioavailability of the growth factor by removal of the C-terminal propeptide, containing a short stretch of positively charged amino acid residues responsible for the retention of the molecule at the cell surface or in the pericellular matrix (La Rochelle *et al.*, 1991; Houck *et al.*, 1992; Keyt *et al.*, 1996a). The resulting effect is similar to that of alternative splicing, which generates polypeptide variants devoid of the retention domain. VEGF-C is secreted readily into the CM, independently of whether it is processed or not. The isolated C-terminal half of VEGF-C is also released efficiently into the CM, when provided with the VEGF-C signal sequence. In addition, VEGF-C does not bind to heparin, which is known to interact with the basic regions of the long VEGF splice isoforms. The short stretch of basic amino acids, located at the C-terminus of the VEGF-C precursor (residues 372–386), either does not affect its secretion or is proteolytically removed. These data suggest that the bioavailability of VEGF-C is not regulated by the same mechanism as in the case of PDGF and VEGF. The propeptides also do not seem to be essential for the folding, assembly or secretion of VEGF-C homodimers, as the ΔNΔC form

was secreted efficiently as a dimer, and possessed all the tested activities of naturally processed VEGF-C.

The major secreted VEGF-C form contains the C-terminal propeptide which has an unusual structure with tandemly repeated cysteine-rich motifs and is linked via disulfide bonds to the N-terminal propeptide. The possible function of this, apparently by itself inactive C-terminal half of VEGF-C is unknown. Besides its striking similarity to the secretory silk protein (BR3P), the C-terminal VEGF-C propeptide also contains short motifs homologous to the EGF-like domains of other secreted proteins, most importantly of the extracellular matrix components such as fibrillin, laminin and tenascin. All of these proteins are known to participate in protein–protein or protein–cell surface interactions (Apella *et al.*, 1988). This observation, together with increasing evidence that the binding of growth factors to the extracellular matrix is a major mechanism regulating growth factor activity (Taipale and Keski-Oja, 1997), suggests that the secreted VEGF-C, which is proteolytically cleaved at the R227/S228 site, may stay associated with the extracellular matrix via its C-terminal propeptide (Figure 10). The unique organization of the BR3 motifs, which differ from previously known repeated motifs in secreted proteins of vertebrata, might provide additional specificity to the VEGF-C association with the extracellular matrix (Figure 10). Cleavage of the N-terminal propeptide would then release the active VEGF-C not only from the latent state, but also from its association with the extracellular matrix (Figure 10). In fact, some similarity can be seen between structural organization of secreted VEGF-C and TGF-β, with the N- and C- terminal VEGF-C propeptides being functional homologues of the TGF-β latency-associated protein and the latent TGF-β-binding protein respectively. The latter has a domain structure and is similar to fibrillin (reviewed in Miyazono *et al.*, 1994; Taipale and Keski-Oja, 1997). The questions of whether secreted VEGF-C indeed remains associated with the extracellular matrix and which protease is responsible for the proteolytic processing of VEGF-C remain to be answered in the future.

## Materials and methods

### *Cell culture, transfections and metabolic labelling*

293-EBNA cells, COS-7 cells, and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (FCS); PC-3 cells in Ham's F12 medium–7% FCS; PAE-KDR, PAE-Flt1 (Waltenberger *et al.*, 1994) and PAE-Flt4 (Pajusola *et al.*, 1994) cells in Ham's F12 medium–10% FCS. BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). Cell transfections were carried out using the calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. When used for stimulation experiments, and for detection of VEGF-C expression by Western blotting, the culture medium was changed to DMEM–0.1% bovine serum albumin (BSA) 48 h after transfection and, after an additional 48 h, this medium was collected, clarified by centrufugation, concentrated using Centriprep-10 or Centricon-10 devices (Amicon) and used in the experiments. Metabolic labelling of non-transfected cells and cells transfected with VEGF-C constructs was carried out by addition of 200 and 100 μCi/ml respectively of Pro-mix™ L-[$^{35}$S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 4 h, the medium was collected or, in some experiments, replaced with DMEM–0.1% BSA, and after an additional incubation for 4 h the media were combined, cleared by centrifugation and used for the immunoprecipitations.

### *Generation of VEGF-C antisera*

Antisera 882 and 905 were generated by immunization of rabbits with synthetic peptides, corresponding to residues 104–120 ($NH_2$-EETIKFAAAHYNTEILK-COOH) and 33–54 ($NH_2$-ESGLDLSDAEPD-AGEATAYASK-COOH). The peptides were synthesized as a branched polylysine structure K3PA4 having four peptide acid chains attached to two available lysine residues. The synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc-chemistry and TentaGel S MAP RAM10 resin mix (RAPP Polymere GmbH). Cleaved peptides were purified by reverse phase HPLC, dissolved in phosphate-buffered saline (PBS), mixed with Freund's adjuvant and used for immunizations of rabbits at bi-weekly intervals according to standard procedures. Antisera obtained after the fourth and fifth booster immunizations were used in the experiments.

### *Immunoprecipitation, Western blotting and analysis of receptor autophosphorylation*

Receptor stimulation, cell lysis, immunoprecipitation and Western blotting followed previously published procedures (Joukov *et al.*, 1996). Immunoprecipitations of metabolically $^{35}$S-labelled VEGF and VEGF-C from CM were carried out using mouse monoclonal anti-human VEGF neutralizing antibody (R&D Systems) and antiserum 882 or 905, respectively. VEGF and VEGF-C bound to antibodies were precipitated using protein G–Sepharose (Pharmacia) and protein A–Sepharose respectively. The peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig (DAKO), diluted 1:1000, and the ECL method (Amersham) were used to detect the polypeptide–antibody complexes on Western blots.

### *Generation of VEGF-C mutants*

VEGF-C mutants were generated using the Altered Sites II *in vitro* Mutagenesis System (Promega). For this purpose, the *Bam*HI fragment of the VEGF-C cDNA from VEGF-C/pREP7 (Joukov *et al.*, 1996) was subcloned in antisense orientation into the pALTER-1 vector. To generate the VEGF-C point mutants, suitable oligonucleotides were synthesized and the mutagenesis procedure was carried out according to the manufacturer's instructions. To generate the N-His construct, an 84mer oligonucleotide was used to introduce the 6×His tag in place of Phe32 (between Ala31 and Glu33). The NT VEGF-C construct was obtained using an oligonucleotide encoding a stop codon instead of Lys214. The deletion mutants were produced by using a loop-out deletion strategy, as described in (Bergman *et al.*, 1995). A 65mer oligonucleotide was used to generate the ΔN VEGF-C construct, in which residues 32–102 of VEGF-C were deleted. In the second round mutagenesis procedure, ΔN VEGF-C and a 52mer oligonucleotide were used to introduce the 6×His tag followed by a stop codon and a *Not*I site immediately after Ile225 to generate ΔNΔC. The CT construct was generated on the basis of the N-His construct. Oligonucleotides (54 and 63mer) were used to introduce *Nco*I sites in the same reading frame, one at the 3′ end of the 6×His tag, and another one at the 5′ end of the sequence encoding the C-terminal part of VEGF-C (starting from Ser228). The resulting construct was subjected to *Nco*I digestion and ligation, giving rise to the construct encoding VEGF-C signal peptide followed by the 6×His tag and the C-terminal half of VEGF-C (additional proline and tryptophan residues were present between the 6×His tag and the C-terminus as a result of introduction of the *Nco*I site in the same reading frame). The mutant constructs in the pALTER vector were digested with *Hin*dIII and *Not*I, subcloned into *Hin*dIII–*Not*I-digested pREP7 and used to transfect 293-EBNA cells.

Strain GS115 of the yeast *P.pastoris* and the expression vector pIC9 (Invitrogen) were used according to the manufacturer's instructions to express ΔNΔC. The VEGF-C sequence was amplified by PCR with a sense primer encoding residues 103–108 and an antisense primer encoding residues 212–215, followed by a 6×His tag. *Eco*RI sites were introduced in the the 5′ and 3′ termini of the sense and antisense primers. The amplified fragment was fused in-frame to the yeast a-factor signal sequence in pIC9.

### *Purification and N-terminal sequence analysis of VEGF-C*

Antibody 882 was employed to purify wt VEGF-C from 1.2 l of CM of transfected 293-EBNA cells by immunoaffinity chromatography. The IgG fraction isolated using protein A–Sepharose (Pharmacia) was covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia) at a concentration of 5 mg of IgG/ml Sepharose resin (Harlow and Lane, 1988). N-His VEGF-C was isolated using Talon™ Metal Affinity Resin (Clontech). Yeast ΔNΔC VEGF-C was purified using Ni-NTA Superflow resin (QIAGEN). No contaminating proteins were detected when 2 μg of the yeast purified ΔNΔC was analysed by SDS–PAGE with subsequent

Coomassie R-250 or silver staining of the gel. The purified material was analysed by electrophoresis, Western blotting and N-terminal amino acid sequence analysis as described earlier (Joukov *et al.*, 1996). An additional sequence obtained during the analysis of the 29–30 kDa polypeptide, $NH_2$-AVVMTQTPAS-COOH, corresponded to the variable region of the Ig-κ chain, which was present in the purified material due to leakage from the affinity matrix.

### *Pulse–chase and dimerization studies*

Metabolic labelling, immunoprecipitation and pulse–chase analysis of polypeptides were done essentially as described previously (Joukov *et al.*, 1996). To study the composition of the VEGF-C dimers, the labelled polypeptide bands electrophoresed under non-reducing conditions were cut out from the gel, soaked for 30 min in 1× gel-loading buffer containing 200 mM β-mercaptoethanol and subjected to a second SDS–PAGE under reducing conditions. Reduction of the disulfide bonds and alkylation of wt, ΔN and R102S VEGF-C were carried out by incubation of CM in the presence of 10 mM dithiothreitol for 2 h at room temperature with subsequent addition of 25 mM iodoacetamide and incubation for 20 min at room temperature.

For polypeptide cross-linking, DSS (Pierce) was added to the serum- and BSA-free CM at a concentration of 1 mM. After incubation for 1 h, the reaction was quenched by addition of 60 mM Tris–HCl (pH 7.4) and incubation was continued for 30 min. The cross-linked VEGF- and VEGF-C complexes were precipitated using anti-VEGF antibodies and antiserum 882, respectively.

### *Binding studies using receptor extracellular domains*

R-3EC (a kind gift from Dr Katri Pajusola) or R-2EC (Cao *et al.*, 1996) were added to the labelled CM, supplemented with 0.5% BSA and 0.02% Tween-20. A similar amount of CM was used for immunoprecipitation with antiserum 882. After incubation for 2 h at room temperature, anti-VEGF-C antibodies and R-3EC protein were absorbed to protein A–Sepharose, and R-2EC was immunoprecipitated using anti-AP monoclonal antibodies (Genzyme) and protein G–Sepharose. The VEGF-C–receptor complexes were washed three times with ice-cold binding buffer (PBS, 0.5% BSA, 0.02% Tween-20) and twice with 20 mM Tris–HCl (pH 7.4). The same media were precipitated using anti-AP antibody and protein G–Sepharose or with protein A–Sepharose to control possible non-specific absorption.

### *Analysis of VEGF-C binding to cell surface receptors*

Mouse recombinant VEGF164 (a kind gift from Dr Herbert Weich) and pure yeast ΔNΔC were labelled with $^{125}I$ using the Iodo-Gen reagent (Pierce), and purified by gel filtration on Sephadex G-15 (Pharmacia). The specific activities were $3.5\times10^6$ c.p.m./pmol and $3.0\times10^6$ c.p.m./pmol for VEGF and ΔNΔC, respectively. Transfected PAE cells grown on gelatinized 24-well plates ($10^5$ cells/well) were washed twice with 0.5 ml of binding buffer (Ham's F12 medium, 25 mM HEPES, pH 7.4, 0.1% BSA, 0.1% sodium azide) and incubated for 1.5 h at room temperature in 0.25 ml of binding buffer with increasing concentrations (in saturation analysis) or with a 100 pM concentration of the labelled factor and increasing concentrations of the non-labelled factor (in competition experiments). The cells were then placed on ice, washed three times with ice-cold PBS/0.1% BSA, lysed in 1 M NaOH and counted in a γ-counter. To estimate the non-specific values in saturation binding, the same determinations were done in the presence of unlabelled ΔNΔC.

In order to cross-link the iodinated factors to the cell surface receptors, cells grown on 10 cm gelatinized cell culture dishes were incubated for 1.5 h at room temperature in binding buffer, containing 400 pM of [$^{125}I$]VEGF or [$^{125}I$]ΔNΔC. After two washes with PBS, the incubation was continued in PBS containing 0.5 mM of [bis(sulfosuccinimidyl)suberate] ($BS^3$) (Pierce) for 30 min at room temperature. Then 50 mM Tris–HCl was added to quench the reaction, the cells were washed twice with Tris-buffered saline and lysed in RIPA buffer. The lysates were subjected to immunoprecipitation with VEGFR-2-specific antibodies, and the precipitated material was analysed by SDS–PAGE and autoradiography.

### *Analysis of VEGF-C biological activity*

Mitogenic assays for VEGF-C were carried out by analysis of thymidine incorporation into BCE cells (Olofsson *et al.*, 1996a) and endothelial cell migration assays in the three-dimensional collagen gel as described in Joukov *et al.* (1996). Vascular permeability was determined by the Miles assay (Miles and Miles, 1952). Briefly, depilated guinea pigs were injected intracardially with 20 mg/kg of Evans Blue (Sigma) in 0.5 ml of isotonic saline. The analysed polypeptides were dissolved in PBS and injected intradermally in a volume of 0.1 ml into the back of guinea pigs. In some experiments, the analysed material was pre-treated with 15 μg/ml of anti-human VEGF neutralizing antibody (R&D systems). After 20 min, the animals were sacrificed under anaesthesia, skin at the injection sites was excised and the amount of extravasated dye was quantitated by elution of the dye with 4.0 ml of formamide for 4 days at 45°C and measuring the optical density of the eluate at 620 nm (Udaka *et al.*, 1970). Similar results were obtained in three separate experiments.

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| INFORMATION DISCLOSURE STATEMENT IN AN APPLICATION (Use several sheets if necessary) | HU et al. | |
| | Filing Date December 23, 1998 | Group Art Unit 1646 |

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| **Application Number** | 09/219,442 |
| **Filing Date** | December 23, 1998 |
| **First Named Inventor** | Hu, et al. |
| Group Art Unit | 1647 |
| Examiner Name | Saoud, C. |
| Attorney Docket Number | **PF112P2D1** |

Sheet 1 of 2

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| Group Art Unit | 1647 |
| Examiner Name | Saoud, C. |
| Attorney Docket Number | **PF112P2D1** |

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